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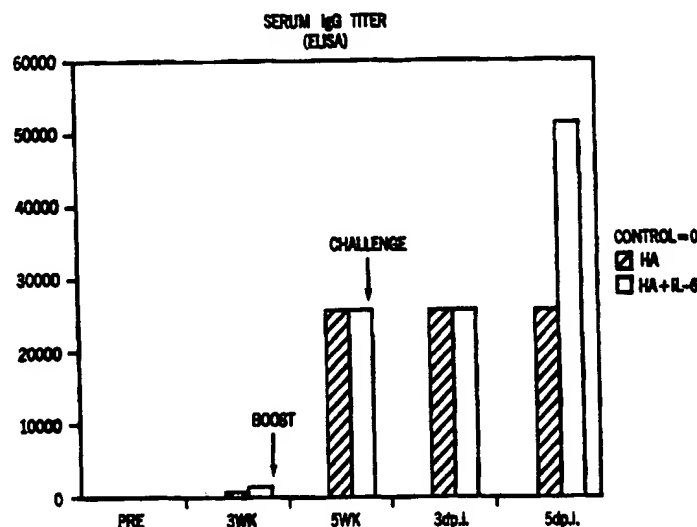
(57) Abstract

    A method of providing a patient with an enhanced immune response is disclosed. In one embodiment, the method comprises the step of vaccinating the patient with a vaccine comprising a combination of DNA encoding interleukin-6 and DNA encoding an antigen capable of enlisting an enhanced immune response in a patient. In one embodiment, the enhanced immune response is a therapeutic response. In another embodiment, the enhanced immune response is a protective immune response.

METHOD OF DNA VACCINATION USING DNA ENCODING ANTIGEN AND ENCODING IL6

This application claims priority to U.S. provisional application 60/052,441 filed July 14, 1997.

STATEMENT REGARDING FEDERALLY SPONSORED
RESEARCH AND DEVELOPMENT

Not applicable.

BACKGROUND OF THE INVENTION

Influenza A viruses are important pathogens in a variety of mammalian and avian species (Murphy, B.R. and R.G. Webster, 1996). In horses, influenza virus infection is a medically and economically important disease throughout the world and is one of the most common causes of equine respiratory disease in North America (Mumford, J., 1992; Traub-Dargatz, J.L., et al., 1991). Two subtypes of influenza A viruses exist in horses. Viruses of the H3N8 (A/equine-2) subtype are the most commonly isolated subtype throughout the world, whereas H7N7 viruses (A/equine-1) have been identified only rarely in the last 15 years [Murphy, B.R. and R.G. Webster, 1996; Singh, G., 1996; Madic, J., et al., 1996].

Intense vaccination programs for horses are employed in an effort to control infection with influenza virus due to the high morbidity and economic losses associated with outbreaks. However, the inactivated, whole virus vaccines that are commercially-available offer only limited short-term protection (Mumford, J., 1992). In

contrast, recovery from natural infection results in
complete immunity to reinfection for at least 6 months
and partial immunity for over one year (Hannant, D., et
al., 1988). Therefore, new vaccination protocols that
5   more closely mimic the responses to natural infection are
clearly needed. Mumford and colleagues have shown that
ISCOMs prepared from purified equine influenza virus are
more highly immunogenic than conventional inactivated
virus vaccines and induce protection from challenge in
10 horses (Hannant, D., et al., 1988; Mumford, J.A., et al.,
1994), but these vaccines are not available in the U.S.
As described below, we have evaluated gene gun-mediated
DNA vaccination as an alternative approach. In
comparison to administration of preformed protein
15 antigen, DNA vaccination is particularly attractive
because the immunogen of interest is actively synthesized
de novo in transfected cells. Therefore, the immunogen
is available for expression by MHC class I as well as MHC
class II molecules (Webster, R.G., et al., 1994; Haynes,
20 J.R., et al., 1996).

      DNA vaccination has been shown previously to elicit
immune responses to a wide variety of viral, bacterial
and protozoal pathogens (Donnelly, J.J., et al., 1994;
Sakaguchi, M., et al., 1996; Whalen, R.G., 1996). In
25 particular, immune responses to avian influenza virus
infection in chickens and human influenza virus infection
in mice and ferrets have been demonstrated following DNA
administration via intravenous, intramuscular, intranasal

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and gene gun-mediated routes of delivery (Webster, R.G., et al., 1994; Ulmer, J.B., et al., 1993; Fynan, E.F., et al., 1993; Ulmer, J.B., et al., 1994). Cutaneous administration of DNA with the gene gun is, however, the

5   most efficient approach, requiring 250-5,000 fold less DNA than parenteral injection techniques (Fynan, E.F., et al., 1995; Pertmer, T.M., et al., 1995). This approach also provides an added safety advantage over intramuscular injection since the administered DNA should

10   be removed from the body through normal epidermal cell turnover (Donnelly, J.J., et al., 1994).

Needed in the art of DNA vaccination is an adjuvant capable of providing an enhanced protective and therapeutic immune response.

15   BRIEF SUMMARY OF THE INVENTION

In one embodiment, the present invention is a method of providing a mammalian patient with an enhanced immune response to a specific antigen. The enhanced immune response may be in the form of either protective

20   antibodies or cellular effectors, such as cytotoxic T-lymphocytes (CTL).

The method comprises the steps of vaccinating the patient with a vaccine comprising a combination of DNA encoding interleukin-6 and DNA encoding an antigen

25   capable of eliciting an immune response in the patient. The DNA encoding both the interleukin-6 and the antigen

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are operably linked to control sequences which direct the expression thereof in the patient.

In one form of the present invention, the interleukin-6 and antigen are encoded on a single nucleic

5   acid construct.

In a preferred form of the invention, the antigen is a viral antigen. Preferably, the virus is one that infects across mucosal surfaces. In a preferred form of the invention, the virus is selected from the group

10   consisting of influenza virus, rotaviruses, herpes viruses and HIV. In another embodiment, the virus may be a blood-borne pathogen, such as hepatitis B virus.

Other forms of the invention encompass antigens specific for bacteria, protozoa, and fungi.

15   Additionally, the present invention is suitable for anti-cancer applications and the antigen is a tumor antigen.

Preferably, the vaccination is via biolistic methods and the preferred vaccination sites are skin and oral or ocular mucosa. An equine vaccination, a preferred site

20   of vaccination is the conjunctiva of the eye.

It is an object of the present invention to provide an enhanced protective antibody immune response.

It is another object of the present invention to provide an enhanced response for cellular effectors, such

25   as CTLs.

It is a feature of the present invention that co-administration of DNA encoding IL-6 with a DNA vaccine enhances the protective immune response of the vaccine.

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Other objects, features and advantages of the
present invention will become apparent after review of
the specification, claims and drawings.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

5   Fig. 1(A) and (B) describe virus-specific serum IgG
(A) and IgA (B), as measured by ELISA, in mice vaccinated
with control pWRG DNA, Eq/KY HA DNA or Eq/KY HA DNA +
IL-6 DNA. Fig. 1(C) describes virus-neutralizing Ab
titers in mice vaccinated with control pWRG DNA, Eq/KY HA

10   DNA or Eq/KY HA DNA + IL-6 DNA.

Fig. 2 shows the ratio of virus-specific IgG1/IgG2a,
as measured by ELISA, in the serum of mice vaccinated
with control pWRG DNA, Eq/KY HA DNA or Eq/KY HA DNA + IL-
6 DNA.

15   Fig. 3 describes virus-specific IgG titers as
measured by ELISA in nasal wash specimens from mice
vaccinated with control pWRG DNA, Eq/KY HA DNA or Eq/KY
HA DNA + IL-6 DNA.

Fig. 4 shows the mean titers (and standard errors of

20   the means) of virus in the lungs of mice following
vaccination with control pWRG DNA, Eq/KY HA DNA, Eq/KY HA
DNA + IL-6 DNA or IL-6 DNA alone. Fig. 4(A) shows the
results from experiment 1 and Fig. 4(B) shows the results
from experiment 2.

DESCRIPTION OF THE INVENTION

A. Definitions

Unless defined otherwise, all technical and
scientific terms used herein have the same meaning as
5 commonly understood by one of ordinary skill in the art
to which the invention pertains. The following terms are
intended to be defined as indicated below.

An "antigen" refers to any agent, generally a
macromolecule, which can elicit an immunological response
10 in an individual. The immunological response may be
mediated by B-and/or T-lymphocytic cells. The term may
be used to refer to an individual macromolecule or to a
homogeneous or heterogeneous population of antigenic
macromolecules. As used herein, the "antigen" is
15 generally used to refer to a protein molecule or portion
thereof which contains one or more epitopes.

A "B cell epitope" generally refers to the site on
an antigen to which a specific antibody molecule binds.
The identification of epitopes which are able to elicit
20 an antibody response is readily accomplished using
techniques well known in the art. See, e.g., Geysen, et
al., 1984) (general method of rapidly synthesizing
peptides to determine the location of immunogenic
epitopes in a given antigen); U.S. patent No. 4,708,871
25 (procedures for identifying and chemically synthesizing
epitopes of antigens); and Geysen, et al., 1986)
(techniques for identifying peptides with high affinity
for a given antibody).

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"T-cell epitopes" are generally those features of a peptide structure capable of inducing a T-cell response. In this regard, it is accepted in the art that T-cell epitopes comprise linear peptide determinants that assume

5   extended conformations within the peptide-binding cleft of MHC molecules, (Unanue, et al., 1987). As used herein, a T-cell epitope is generally a peptide having about 3-5, preferably 5-10 or more amino acid residues.

"Gene delivery" refers to methods or systems for

10   reliably delivering foreign DNA into host cells. Such methods can result in the expression of the foreign DNA in the host cells.

A "nucleotide sequence" or a "nucleic acid molecule" refers to single- or double-stranded DNA and RNA

15   sequences. The term captures molecules that include any of the known base analogues of DNA and RNA.

A "coding sequence" or a sequence which "encodes" a particular polypeptide antigen, is a nucleic acid sequence which is transcribed (in the case of NDA) and

20   translated (in the case of mRNA) into a polypeptide *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences.

The term DNA "regulatory sequences" refers collectively to promoter sequences, polyadenylation

25   signals, transcription termination sequences, upstream regulatory domains, origins of replication, internal ribosome entry sites ("IRES"), enhancers, and the like, which collectively provide for the transcription and

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translation of a coding sequence in a recipient cell.
Not all of these control sequences need always be present
so long as the selected gene is capable of being
transcribed and translated in an appropriate recipient

5   cell. The control sequences for eukaryotes and
prokaryotes can differ significantly, and for the present
invention eukaryotic, and preferably, mammalian or
mammalian virus control sequences are most suitable.

"Operably linked" refers to an arrangement of

10   elements wherein the components so described are
configured so as to perform their usual function. Thus,
control sequences operably linked to a coding sequence
are capable of affecting the expression of the coding
sequence. The control sequences need not be contiguous

15   with the coding sequence, so long as they function to
direct the expression thereof. Thus, for example,
intervening untranslated yet transcribed sequences can be
present between a promoter sequence and the coding
sequence and the promoter sequence can still be

20   considered "operably linked" to the coding sequence.

B.   General Methods

Before describing the present invention in detail,
it is to be understood that this invention is not limited
to particular formulations or process parameters as such

25   may, of course, vary. It is also to be understood that
the terminology used herein is for the purpose of
describing particular embodiments of the invention only,
and is not intended to be limiting.

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Although a number of methods and materials similar or equivalent to those described herein can be used in the practice of the present invention, the preferred materials and methods are described herein.

5   The present invention provides a method for eliciting in a mammalian subject, an immune response against a selected antigen using nucleic acid immunization and techniques. The method can thus be used in a variety of mammalian subjects to provide a suitable

10   therapeutic or prophylactic immune response against infection or disease. Suitable antigens include those derived from Human Pappiloma Viruses (HPV), HIV, HSV2/HSV1, influenza virus (types A, B, and C), Polio virus, RSV virus, Rhinoviruses, Rotaviruses, Hepatitis A

15   virus, Norwalk Virus Group, Enteroviruses, Astroviruses, Measles virus, Para Influenza virus, Mumps virus, Varicella-Zoster virus, Cytomegalovirus, Epstein-Barr virus, Adenoviruses, Rubella virus, Human T-cell Lymphoma type I virus (HTLV-I), Hepatitis B virus (HBV), Hepatitis

20   C virus (HCV), Hepatitis D virus, Px virus, Marbug and Ebola; bacteria including *M. tuberculosis*, *Chlamydia*, *N. Gonorrhea*, *Shigella*, *Salmonella*, *Vibrio Cholera*, *Treponema pallidua*, *Pseudomonas*, *Bordetella pertusis*, *Brucella*, *Franciscella tulorensis*, *Helicobacter pylori*,

25   *Leptospria interrogaus*, *Legionella pneomophila*, *Yersinina pestis*, *Streptococcus* (types A and B), *Pneumococcus*, *Meningococcus*, *Hemophilus influenza* (type B), *Toxoplasma gondic*, *Complylobacteriosis*, *Moraxella catarrhalis*,

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Legionella pneumophlia, Pseudomonas aeruginosa,
Donovanosis and *Actinomycosis;* fungal pathogens including
Candidiasis and Aspergillosis, parasitic pathogens
including Taenia, Flukes, Roundworms, Amebiasis,

5   Giardiasis, Cryptosporidium, Schistosoma, Pneumocystis
carinii, Trichomoniasis and Trichinosis. The present
invention can be used to provide a suitable immune
response against numerous veterinary diseases, such as
Foot and Mouth diseases, Coronavirus, *Pasteurella*

10   *multocida, Helicobacter, Strongylus vulgaris,*
Actinobacillus pleuropneumonia, Bovine viral virus
diarrhea (BVDV), *Klebsiella pneumoniae, E. coli,*
Bordetella pertussis, Bordetella parapertussis and
brochiseptica.

15   C.   Isolation of Genes and Construction of Vectors
Nucleotide sequences selected for use in the present
invention can be derived from known sources, for example,
by isolating the same from infected cells or viral
particles containing a desired gene or nucleotide

20   sequence using standard techniques. The nucleotide
sequences for many, if not most, pathogen antigens have
been identified to assist in vaccine and therapy design.
It is now possible to construct DNA molecules of
significant length once DNA sequence information is

25   available.
Once coding sequences for desired antigens have been
prepared or isolated, such sequences can be cloned into
any suitable vector or replicon. Numerous cloning

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vectors are known to those of skill in the art, and the

selection of an appropriate cloning vector is a matter of

choice. Ligations to other sequences are performed using

standard procedures, known in the art.

5   Selected nucleotide sequences can be placed under

the control of regulatory sequence such as a promoter or

ribosome binding site (also referred to herein as

"control" elements), so that the sequence encoding the

desired antigen is transcribed into RNA in the host

10   tissue transformed by a vector containing this expression

construct.

The choice of control elements will depend on the

host being treated and the type of preparation used.

Thus, if the host's endogenous transcription and

15   translation machinery will be used to express the

proteins, control elements compatible with the particular

host will be utilized. In this regard, several promoters

for use in mammalian systems are known in the art and

include, but are not limited to, promoters derived from

20   SV40, CMV, HSV, RSV, MMTV, among others.

In addition to control sequences, it may be

desirable to add regulatory sequences which allow for

regulation of the expression of antigens encoded by the

delivered nucleotide sequences. Regulatory sequences are

25   known to those of skill in the art, and examples include

those which cause the expression of a coding sequence to

be turned on or off in response to a chemical of physical

stimulus, including the presence of a regulatory

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compound. Other types of regulatory elements may also be present in the vector, for example, enhancer sequences.

An expression vector is constructed so that the particular coding sequence is located in the vector with

5 the appropriate control and, optionally, regulatory sequences such that the positioning and orientation of the coding sequence with respect to the control sequences allows the coding sequence to be transcribed under the "control" of the control sequences (i.e., RNA polymerase,

10 which binds to the DNA molecule at the control sequences, transcribes the coding sequence). Modification of the sequences encoding the particular antigen of interest may be desirable to achieve this end. For example, in some cases it may be necessary to modify the sequence so that

15 it is attached to the control sequences with the appropriate orientation; i.e., to maintain the reading frame. The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector. Alternatively, the coding

20 sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site.

Conventional mammalian expression vectors and elements can be enhanced for use as DNA vaccines. For

25 example, it has been found that the addition of signal peptide sequences directing secretion of expressed proteins can enhance CTL immune response. The use of a Kozak ATG sequence can enhance the translational

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efficiency of a DNA vaccine. The inclusion of a
mono/poly ubiquitination sequence in the expression
vector can enhance the MHC Class I presentation signal
while alternatively the use of an invariant chain
5   sequence can enhance MHC Class II presentation signal.
The use of such elements is within the abilities of those
of skill in the art.

D.   Administration of Nucleic Acid Preparations

Particle-mediated methods for delivering nucleic
10   acid preparations are known in the art. Thus, once
prepared and suitably purified, the above-described
nucleic acid molecules can be coated onto carrier
particles using a variety of techniques known in the art.
Carrier particles are selected from materials which have
15   a suitable density in the range of particle sizes
typically used for intracellular delivery from a gene gun
device. The optimum carrier particle size will, of
course, depend on the diameter of the target cells.

For the purpose of the invention, tungsten, gold,
20   platinum and iridium carrier particles can be used.
Tungsten and gold particles are preferred. Tungsten
particles are readily available in average sizes of 0.5
to 2.0 um in diameter. Although such particles have
optimal density for use in particular acceleration
25   delivery methods, and allow highly efficient coating with
DNA, tungsten may potentially be toxic to certain cell
types and may degrade DNA over time. Gold particles or
microcrystalline gold (e.g., gold powder A1570, available

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from Engelhard Corp., East Newark, NJ) will also find use
with the present methods. Gold particles provide
uniformity in size (available from Alpha Chemicals in
particle sizes of 1-3 um, or available from Degussa,

5   South Plainfield, NJ in a range of particle sizes
including 0.95 um) and reduced toxicity.
Microcrystalline gold provides a diverse particle size
distribution, typically in the range of 0.5-5um.

A number of methods are known and have been

10   described for coating or precipitating DNA or RNA onto
gold or tungsten particles. Most such methods generally
combine a predetermined amount of gold or tungsten with
plasmid DNA, $CaCl_2$ and spermidine. The resulting solution
is vortexed continually during the-coating procedure to

15   ensure uniformity of the reaction mixture. After
precipitation of the nucleic acid, the coated particles
can be transferred to suitable membranes and allowed to
dry prior to use, coated onto surfaces of a sample module
or cassette, or loaded into a delivery cassette for use

20   in particular gene gun instruments.

E.   Administration of Coated Particles

Following their formation, carrier particles coated
with either nucleic acid preparations, or peptide or
protein antigen preparations, are delivered to mucosal

25   tissue using particle-mediated delivery techniques.

Various particle acceleration devices suitable for
particle-mediated delivery are known in the art, and are
all suited for use in the practice of the invention.

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Current particle acceleration device designs employ an explosive, electric or gaseous discharge to propel coated carrier particles toward target cells. The coated carrier particles can themselves be releasably attached

5   to a movable carrier sheet, or removably attached to a surface along which a gas stream passes, lifting the particles from the surface and accelerating them toward the target. An example of a gaseous discharge device is described in U.S. Patent No. 5,204,253. An explosive-

10   type device is described in U.S. Patent No. 4,945,050. One example of an electric discharge-type particle acceleration apparatus is the instrument (PowderJect Vaccines, Inc., Madison, WI) , which instrument is described in U.S. Patent No. 5,120,657. Another electric

15   discharge apparatus suitable for use herein is described in U.S. Patent No. 5,149,655. The disclosure of all of these patents is incorporated herein by reference in their entireties.

      The coated particles are administered to the subject

20   to be treated in a manner compatible with the dosage formulation, and in an amount that will be effective to bring about a desired immune response. The amount of the composition to be delivered which, in the case of nucleic acid molecules is generally in the range of from 0.001 to

25   10.0 ug, more preferably 0.25 to 5.0 ug of nucleic acid molecule per dose, depends on the subject to be treated. By dose, it is meant to refer to a single event of delivery , for example, by gene gun. Using current gene

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guns, it is common for a single immunization procedure, whether a prime immunization or a boost, to include more than one gene gun dose. For example, a prime might consist of two to six gene gun doses to the tongue.

5   Adding more DNA to each dose, beyond 0.25 to 5ug, generally does not increase immune response. The additional doses are appropriate to, in essence, treat more tissue. A gene gun design which is capable of treating more tissue in a single operation would lower

10   the number of doses in single vaccination. In general, however, the total amount of DNA delivered in the entire immunization will be in the range of about 1-30ug total for all doses. Often a prime immunization and either one or two boost immunizations will be appropriate to achieve

15   the desired level of immune response. The exact amount necessary will vary depending on the age and general condition of the individual being immunized and the particular nucleotide sequence or peptide antigens selected, as well as other factors. An appropriate

20   effective amount can be readily determined by one of skill in the art upon reading the instant specification.

F.   In General

The experiments described below were conducted to investigate whether co-administration of DNA encoding

25   human interleukin-6 (IL-6) would enhance immune responses to a hemagglutinin (HA) or hepatitis DNA vaccine in model mouse systems for both viruses and an equine system for influenza virus. The experiments showed that influenza

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virus-specific serum IgA and nasal IgG appeared more
rapidly and serum virus neutralizing antibody titers were
higher prior to challenge in the mice vaccinated with HA
+ IL-6 DNA compared to those that received HA DNA alone.

5   In horses, co-administration of HA and equine IL-6 DNA
enhanced influenza virus-specific serum IgG antibody
responses with a broadening of the responses to include
the IgG(T) isotype, IgA responses in nasal secretions,
and lymphocyte proliferation responses in the lymph nodes

10   draining the site of DNA vaccination.

In a similar fashion, mice vaccinated with a
hepatitis B virus DNA vaccine and IL-6 DNA demonstrated
enhanced immune responses compared to those receiving
hepatitis B virus DNA alone. Specifically, there was an

15   increase in CTL responses with mice immunized by co-
delivery of IL-6.

Following homologous influenza virus challenge
infection, mice that had received HA DNA alone had
reduced levels of virus in their lungs and cleared their

20   infections more rapidly than controls, but they were not
protected from infection. In contrast, mice that
received both HA and IL-6 DNA were protected, as
evidenced by a lack of detectable virus in their lungs
after challenge. These results support the use of IL-6

25   as a cytokine adjuvant to DNA vaccination.

Therefore, in one embodiment, the present invention
is a method of enhancing protective immune responses by
co-administering DNA encoding interleukin-6, with a DNA

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vaccine. This protective immune response is most easily measured by virus-specific antibody responses in serum. or in mucosal secretions and by prevention or reduction of virus replication.

5   The method of the present invention provides that following virus infection, the patients receiving both the DNA vaccine and IL-6 will have reduced detectable virus, preferably tested in lungs for influenza virus. Most preferably, the vaccine recipient will be completely

10   protected as evidenced by a total lack of detectable virus.

Our results below also show that the vaccinated patient will show an increased cytotoxic T-lymphocytes (CTL) levels. Our Examples below demonstrate that levels

15   of CTL are greater. than or equal to 45% higher in mice immunized with the vaccine plus IL-6 than in mice immunized with only the vaccine.

Preferably, the method of vaccination is via particle-mediated gene transfer methods, such as the

20   PowderJect gene delivery device. Other methods of DNA vaccination suitable for the present invention are summarized in Fynan, E.F., et al., 1993; Donnelly, J.J., et al.,1994; Fynan, E.F., et al., 1995; Liu, M.A., 1995 (entire volume was devoted to DNA vaccination); Robinson,

25   H.L., et al., 1996; Ulmer, J.B., et al., 1996; and Donnelly, J.J., et al., 1996).

Preferred sites of immunization are the skin surface of the patient and the oral or ocular mucosal surfaces.

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Preferably, the DNA vaccine comprises an antigen capable of eliciting a protective immune response in the patient.

In one preferred embodiment, the DNA vaccine would

5   comprise the influenza hemagglutinin gene, the hepatitis surface antigen, or other genes from viruses that infect across mucosal surfaces. Therefore, we envision that viruses such as influenza viruses, HIV, rotaviruses and herpes viruses are especially suitable for the present

10   invention.

In another embodiment of the present invention, one may wish to immunize against blood-borne pathogens, such as hepatitis B virus.

In another embodiment, the antigen is specific for a

15   bacterial, protazoan, or fungal protein and the vaccine is designed to elicit immune response against these pathogens.

In another embodiment of the invention, the antigen is designed to provoke an anti-cancer response. Examples

20   of such an antigen are gp100, Mart-1, Epcam-1 and MuC-1. In this embodiment of the present invention, one would immunize a tumor patient and invoke a therapeutic CTL response.

We envision that mammals, such as horses, pigs,

25   cows, and humans, and avian species, such as birds, can be successfully vaccinated by the present invention.

Preferably, one would use a human interleukin-6 gene when vaccinating humans and an equine interleukin-6 gene

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when vaccinating horses. However, our studies show that heterologous genes are also useful.

The examples below disclose preferred methods of co-administering the IL-6 DNA and vaccine. Other methods of

5   preparing these reagents will be apparent to one of skill in the art of microbiology.

<div align="center">EXAMPLES</div>

1.   <u>Use of DNA Encoding Human IL-6 as an adjuvant to
     influenza virus HA DNA Vaccination in Mice (as</u>
10   <u>described in Larsen, et al., 1998, incorporated by
     reference)</u>

A.   <u>In General</u>

We have chosen the hemagglutinin (HA) protein as our immunogen because it is the viral protein that contains

15   the major antigenic sites of the virus and it is the protein to which virus neutralizing (VN) antibodies are directed. The HA protein is responsible for virus binding to cells and fusion of the virus envelope and cell membrane to initiate infection (Murphy, B.R. and

20   R.G. Webster, 1996). We have demonstrated previously that ACCELL gene gun-mediated DNA vaccination using the HA gene of A/Equine/Kentucky/1/81 (H3N8) (Eq/KY) virus induces virus-specific antibodies (Abs), including VN Abs, in mice (Olsen, C.W., <u>et al.</u>, 1997). However, only

25   partial protection from challenge infection was achieved unless a very prolonged time period was provided between doses of vaccine (Olsen, C.W., <u>et al.</u>, 1997). In contrast, recovery of mice from a previous infection with

<div align="center">-20-</div>

Eq/KY virus conferred complete immunity to homologous
virus challenge (Olsen, C.W., et al., 1997).

In an attempt to mimic the host response to natural
infection, we hypothesized that addition of a cytokine

5   adjuvant would enhance the immune responses generated by
our HA DNA vaccine and subsequent protection from
infection. Previous studies have investigated the use of
cytokines as vaccine adjuvants. Interleukins-1, -3, -4,
-6, -7 and -12, as well as IFN-gamma, GM-CSF and TNF-

10 alpha have been administered in protein form (Lin, R., et
al., 1995; Lofthouse, S.A., et al. 1995; Noll, A., et
al., 1996; Pockley, A.G. and P.C. Montgomery, 1991) and
IL-5 and -6 have been expressed from recombinant vaccinia
virus vectors (Ramsay, A.J., et al., 1994; Ramsay, A.J.,

15 et al., 1993). In addition, IL-2, IL-8 and GM-CSF have
been expressed from plasmid DNA (Hengge, U.R., et al.,
1996; Xiang, Z. and H.C. Ertl, 1995; Chow, Y.H., et al.,
1997). Our study is unique in its use of IL-6 DNA as a
vaccine adjuvant administered by gene gun delivery.

20 Interleukin-6 is a critical factor in end stage
differentiation of B-cells into IgA secreting plasma
cells (McGhee, J.R. and H. Kiyono, 1992; Holmgren, J., et
al., 1992) and studies in IL-6 knockout mice demonstrated
that IL-6 is vital for maintenance of mucosal IgA

25 responses (Ramsay, A.J., et al., 1994). However, IL-6
also stimulates proliferation of T-cells (Van Snick, J.,
1990). Immunity to influenza is similarly thought to be
dependent upon both local IgA responses for protection at

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the mucosal surfaces and cellular immune responses for clearance of virus from the body (Murphy, B.R. and R.G. Webster, 1996). Our results demonstrate that administration of DNA encoding IL-6 as an adjuvant to HA

5   DNA vaccination confers complete protection from pulmonary infection with influenza virus in mice.

B.   Materials and Methods

Influenza virus and DNA vaccine preparation. A/Equine/Kentucky/1/81, a prototypical H3N8 equine

10   influenza virus, was obtained from the influenza repository at the University of Wisconsin-Madison. The virus was propagated in the allantoic cavity of ten-day-old embryonated chicken eggs as previously described (Olsen, C.W., et al., 1993) and was not

15   specifically adapted for replication in mice. The Eq/KY HA gene was cloned and sequenced previously (Olsen, C.W., et al., 1997). A cDNA encoding human IL-6 (huIL-6) was kindly provided by Dr. Robert Fenton (National Cancer Institute). Human IL-6 is known to bind to murine IL-6

20   (muIL-6) receptors and to be functional in the mouse (Van Snick, J., 1990). Its use in this study allowed us to distinguish between serum IL-6 activity expressed from our DNA construct versus endogenous muIL-6.

The cDNAs for both the Eq/KY HA and huIL-6 were

25   cloned into a CMV promoter-based eukaryotic expression vector (pWRG, PowderJect®, Madison, WI) containing the intron A from CMV, the kanamycin resistance gene and a poly A signal (Olsen, C.W., et al., 1997). Hemagglutinin

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protein expression from the resulting plasmid was confirmed by immunofluorescent antibody staining of transiently transfected Madin-Darby canine kidney (MDCK) cells as previously described (Olsen, C.W., et al.,

5   1997). Interleukin-6 expression was confirmed by testing the supernatant of transiently transfected MDCK cells using both a commercially-available ELISA kit (see below) and the B9 cell assay for IL-6 bioactivity (Aarden, L.A., et al., 1987). Plasmid DNA was prepared for gene gun

10   administration by anion-exchange chromatography (Qiagen, Inc, Chatsworth, CA), adsorbed to gold beads and the beads coated into Tefzel plastic tubing as previously described (Olsen, C.W., et al., 1997; Haynes, J.R., et al., 1996). All DNA cloning procedures throughout this

15   project were conducted using standard techniques (Ausubel, F.M., et al., 1989).

Vaccination protocols. DNA was administered using the PowderJect®XR gene delivery device. Two doses of 2.5 μg of DNA were administered into the epidermis of BALB/c

20   mice, with a three week interval between vaccinations. The mice were divided into three vaccination groups. One group of mice (n=10 mice) received only pWRG plasmid DNA and served as controls. The second group of mice (n=20 mice) received HA + control pWRG DNA and the third group

25   of mice received HA + IL-6 DNA (n=20 mice). The reason for including control pWRG DNA in the HA group was to equilibrate the amount of DNA given and to account for any promoter competition that may occur in the mice

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receiving HA + IL6 DNA. However, for the sake of
clarity, this HA + pWRG combination is hereafter referred
as HA DNA alone.

To confirm our initial protection from challenge
5   results and to obtain samples for assessment of mucosal
immune responses, a second round of similar experiments
was conducted, this time including a fourth group of mice
that received IL-6 + pWRG DNA. This group was included
as an additional control to rule out any direct
10   protective effect of IL-6 in the absence of HA
expression. Two weeks after the second vaccinations, all
mice in the first experiment and half of the mice in each
vaccination group in the second experiment were
challenge-infected with $1 \times 10^{7.4}$ egg infectious dose$_{50}$
15   (EID$_{50}$) units of Eq/KY virus by intranasal instillation
under light Metofane (Pittman Moore, Mundelein, IL)
sedation. The remaining mice in the second experiment
were euthanized 2 weeks after their second vaccination to
obtain nasal wash samples for assessment of mucosal
20   immune responses (see below and Fig. 3) in the absence of
a challenge infection. Challenged mice were euthanized
either 3 or 5 days after infection in experiment 1.
(Half of the mice in each group were euthanized on each
day.) All challenged mice in experiment 2 were
25   euthanized 3 days after infection.

Interleukin 6 assays. Levels of both huIL-6 and
muIL-6 were determined on serum samples obtained 44 hours
after the first and second vaccinations, using

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commercially-available ELISA kits (R&D Systems Inc.,
Minneapolis, MN). The assays were conducted as per the
manufacturer's guidelines.

Samples for virus-specific Ab testing. Blood was
5   collected from the supraorbital sinus for serologic
testing immediately prior to the first vaccinations,
immediately prior to the second vaccinations (3 weeks),
immediately prior to challenge (5 weeks) and at the time
of euthanasia. Blood samples from all of the mice in
10   each vaccination group were pooled at the time of
collection.

In the second experiment, nasal wash samples were
collected to allow for assessment of local mucosal Ab
responses in the upper airways. These samples were
15   obtained 2 weeks after the second vaccination in mice
that were not challenged and 3 days after infection in
the challenged mice. Nasal washes were obtained by
inserting a 22 gauge intravenous catheter retrograde from
the tracheal bifurcation toward the head, positioning the
20   end of the catheter at the caudal area of the nasal
turbinates. One milliliter of sterile PBS +1% BSA was
flushed through the catheter and collected as it drained
from the nares into a sterile petri dish. The flush was
repeated three times using the same volume of fluid.

25   As an additional measure of mucosal immune responses
in the second experiment, virus-specific Abs were
measured in fecal pellets that were collected immediately
prior to challenge and at the time of euthanasia. One

-25-

fecal pellet was collected per mouse and homogenized to a

concentration of 1 mg/ml (W/V) in PBS containing 5% fetal

bovine serum and 0.01% Tween 20.

ELISA and VN Ab assays. Virus-specific Abs were

5   measured in serum, nasal washes and fecal suspensions

using an isotype-specific ELISA assay as previously

described (Olsen, C.W., et al., 1997). Appropriate

positive and negative controls were included on each

plate. The ELISA Ab titers were defined as the

10   reciprocal of the highest dilution of sample for which

the optical density (OD) was a least 2 times the OD of

the negative control sample on that plate. The level of

VN antibodies in serum was determined by inoculation (in

triplicate) of Madin-Darby canine kidney cells with

15   serial dilutions of serum incubated with 50 tissue

culture infectious dose$_{50}$ (TCID$_{50}$) units of Eq/KY virus, as

described previously (Olsen, C.W., et al., 1997). The VN

Ab titers were calculated as the reciprocal of the

highest dilution of serum that completely inhibited Eq/KY

20   virus-induced cytopathic effect.

Virus titration. Lung tissue samples were

homogenized in viral transport media using a Stomacher 80

lab blender (Tekmar Co., Cincinnati, OH). The level of

infectious virus was determined by inoculation (in

25   triplicate) of serial dilutions of each sample into the

allantoic cavity of ten-day-old embryonated chicken eggs.

After incubation at 35°C for 72 hours, a sample of

allantoic fluid from each egg was tested by

hemagglutination assay (Palmer, D.F., et al., 1975) for
the presence of virus. Virus titers were calculated in
EID$_{50}$ units/g lung tissue by the method of Reed and Muench
(Reed, L.J. and H. Muench, 1938). The titers of virus in

5   the lungs of mice in each treatment group were compared
statistically by one-way analysis of variance (ANOVA) and
pairwise contrasts.

C.   Results

Serum IL-6 levels. The levels of human and murine

10   IL-6 were determined in serum samples obtained 44 hours
after DNA vaccinations. Following the first
vaccinations, the level of huIL-6 in serum was 40 pg/ml
in the mice that received IL-6 DNA, but was below the
level of detection (<3 pg/ml) in the mice that received

15   control or HA DNA alone. Following the second
vaccinations, huIL-6 levels were below the level of
detection in all mice. In addition, muIL-6 levels
remained below the level of detection (<15.6 pg/ml) in
all samples, indicating that expression of huIL-6 by DNA

20   administration did not up-regulate endogenous muIL-6
expression.

Virus-specific serum IgG, IgA and VN Ab responses.

Fig. 1(A) and (B) describe virus-specific serum IgG
(A) and IgA (B), as measured by ELISA, in mice vaccinated

25   with control pWRG DNA, Eq/KY HA DNA or Eq/KY HA DNA +
IL-6 DNA. The ELISA assays were performed as previously
described (Olsen, C.W., et al., 1997). ELISA titers on
the Y-axis are defined as the reciprocal of the highest

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dilution of serum for which the OD was a least 2 times the OD of the negative control sample on that plate. Fig. 1C describes virus-neutralizing Ab titers in mice vaccinated with control pWRG DNA, Eq/KY HA DNA or Eq/KY

5   HA DNA + IL-6 DNA. Virus neutralizing Abs were measured by inoculation (in triplicate) of Madin-Darby canine kidney cells with serial dilutions of serum incubated with 50 TCID$_{50}$ units of Eq/KY virus. (See the Materials and Methods.) The VN titers on the Y-axis are defined as

10   the reciprocal of the highest dilution of serum that completely inhibited Eq/KY virus-induced cytopathic effect.

The times of sampling are shown on the X-axis. The times of second vaccination (boost) and challenge

15   infection are indicated by arrows.

Administration of HA and HA + IL-6 DNA induced Eq/Ky virus-specific serum IgG and VN Abs and primed the mice for production of virus-specific serum IgA following challenge. No antibodies to Eq/KY virus were detectable

20   in the control mice that received pWRG control DNA or IL-6 DNA in the absence of HA DNA. Virus-specific IgG levels were negligible 3 weeks after the first vaccination, but rose to titers of 25,000 by 2 weeks after the second vaccination (immediately prior to

25   challenge) in the mice that received either HA or HA + IL-6 DNA (Fig. 1A). Virus-specific IgA was not detectable until after challenge infection in either group of mice. Following challenge, IgA appeared earlier

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in the mice that received HA + IL-6 DNA and rose to a

higher titer (1 serum dilution) by 5 days after

challenge, compared to the mice that were vaccinated with

HA DNA alone (Fig. 1B).

5   The kinetics of the VN Ab responses paralleled those

of the serum IgG responses. At the time of challenge,

the VN titer of the mice that received HA + IL-6 DNA was

50% higher than the titer in the mice that received HA

DNA alone.

10   *Virus-specific IgG subclass responses.* In the

mouse, the Th1 pathway favors the production of Abs of

the IgG2a isotype and the Th2 pathway favors IgG1

production (Mosmann, T.R. and S. Sad, 1996; Finkelman,

F.D., et al., 1990). We examined the ratio of IgG1 to

15   IgG2a immediately prior to and following challenge as an

indirect reflection of Th1/Th2 responses. Fig. 2 shows

the ratio of virus-specific IgG1/IgG2a, as measured by

ELISA, in the serum of mice vaccinated with control pWRG

DNA, Eq/KY HA DNA or Eq/KY HA DNA + IL-6 DNA. The ELISA

20   assays were performed using isotype-specific, horseradish

peroxidase-conjugated, rabbit anti-mouse Ab as described

previously (Olsen, C.W., et al., 1997). The IgG1 and

IgG2a titers used to calculate the ratios shown on the

Y-axis were defined as the reciprocal of the serum

25   dilutions for which the ODs were a least 2 times the OD

of the negative control sample on that plate. The times

of sampling are shown on the X-axis. The times of second

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vaccination (boost) and challenge infection are indicated by arrows.

IgG1 responses predominated in all mice and the IgG1/2a ratios were largely similar in both the HA and HA
5   + IL-6 DNA vaccinated mice. The only detectable difference was a 2-fold higher IgG1/2a ratio at the time of and 3 days after challenge in the mice vaccinated with HA DNA alone (Fig. 2).

Virus-specific mucosal IgG and IgA responses. In
10   the second experiment, we assessed mucosal IgG and IgA responses to our DNA vaccination regimes. No virus-specific IgA was detectable in any of the nasal washes or fecal pellets tested. However, virus-specific IgG was detected in the nasal washes. In particular,
15   virus-specific IgG was present in the nasal washes prior to challenge in the mice vaccinated with HA + IL-6 DNA, whereas it was not detectable until after challenge infection in the mice that received HA DNA alone. In addition, by 5 days after challenge, the virus-specific
20   IgG titer was 4-fold higher in the mice vaccinated with HA + IL-6 DNA (Fig. 3).

Fig. 3 describes virus-specific IgG titers, as measured by ELISA, in nasal wash specimens from mice vaccinated with control pWRG DNA, Eq/KY HA DNA or Eq/KY
25   HA DNA + IL-6 DNA. The ELISA assay was performed as described previously (Olsen, C.W., *et al.*, 1997). Titers on the Y-axis are defined as the reciprocal of the highest dilution of sample for which the OD was a least 2

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times the OD of the negative control sample on that
plate. The times of sampling are shown on the X-axis.
The times of second vaccination (boost) and challenge
infection are indicated by arrows.

5   *Protection from challenge infection.* Fig. 4 shows
the mean titers (and standard errors of the means) of
virus in the lungs of mice following vaccination with
control pWRG DNA, Eq/KY HA DNA , Eq/KY HA DNA + IL-6 DNA
or IL-6 DNA alone. Mice were challenged with 1 x $10^{7.4}$

10 EID_{50} units of Eq/KY virus by intranasal instillation
under light Metofane (Pittman Moore) sedation, two weeks
after the second vaccinations. Fig. 4(A) shows the
results from experiment 1 and Fig. 4(B) shows the results
from experiment 2. Virus was detected by inoculation (in

15 triplicate) of serial dilutions of each of the mouse
lungs into the allantoic cavity of ten-day-old
embryonated chicken eggs (see the Materials and Methods).
Mice were euthanized for collection of lung samples
either 3 or 5 days after challenge, as shown on the

20 X-axises.

In experiment 1 (Fig. 4A), compared to the control
vaccinated mice, the mice that received HA DNA had
reduced levels of virus in their lungs by day 3 after
challenge and had cleared their infections by 5 days

25 after challenge. However, the mice vaccinated with
HA+IL-6 DNA were completely protected from pulmonary
infection, as evidenced by a lack of virus in their lungs
as early as 3 days after challenge. The difference in

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virus titers between the HA and HA + IL-6 DNA vaccinated
mice 3 days after challenge is highly statistically
significant (p<0.0001). These results were confirmed in
a second experiment that also included a group of mice

5 . that were given IL-6 DNA alone (Fig. 4B). Once again,
the mice that received both HA + IL-6 DNA were completely
protected. The virus titers in the mice that received
IL-6 DNA without HA DNA were comparable to those in the
control DNA vaccinated mice, thus confirming that the

10   protection we observed cannot be attributed to any effect
of IL-6 in the absence of co-administered specific
antigen.

D.   Discussion

Infection with equine influenza virus is the most

15   common respiratory illness of horses in North America
(Mumford, J., 1992; Traub-Dargatz, J.L., et al., 1991).
Unfortunately, commercially-available inactivated virus
vaccines offer only very limited, short-term protection,
(Traub-Dargatz, J.L., et al., 1991) although recovery of

20   horses from natural infection leads to long-lasting
protection (Hannant, D., et al., 1988). Likewise, we
have previously demonstrated that recovery from Eq/KY
infection in BALB/c mice confers complete immunity to
subsequent challenge infection (Olsen, C.W., et al.,

25   1997). However, in those initial studies, Eq/KY HA DNA
vaccination could not induce a similar level of complete
protection unless a very prolonged time period was
provided between doses of vaccine (Olsen, C.W., et al.,

-32-

1997). In the present study, we have investigated whether co-administration of DNA encoding the huIL-6 gene would enhance the efficacy of HA DNA vaccination. Our results clearly indicate that gene gun co-administration

5   of HA and IL-6 DNA confers much greater protection from pulmonary infection with Eq/KY virus in mice than does administration of HA DNA alone. The mice that received HA + IL-6 DNA were completely protected from pulmonary infection, with no detectable virus in their lungs after

10   challenge (Figs. 4A and 4B).

A number of cytokines have been investigated previously as vaccine adjuvants, including IL-1, -2, -3, -4, -5, -6, -7, -8 and -12, IFN-gamma, GM-CSF and TNF-alpha (Lin, R., et al., 1995; Lofthouse, S.A., et al.,

15   1995; Noll, A. and I.B. Autenrieth, 1996; Pockley, A.G. and P.C. Montgomery, 1991; Ramsay, A.J., et al., 1994; Ramsay, A.J., et al., 1993; Hengge, U.R., et al., 1996, Xiang, Z. and H.C. Ertl, 1995; Chow, Y.H., et al., 1997; Geissler, M., et al., 1997). We specifically chose to

20   use IL-6 because of its pleiotropic effects on both the humoral and cellular components of the immune system. Interleukin-6 functions as a co-stimulatory molecule for T-cell proliferation (Van Snick, J., 1990) and it enhances B cell differentiation and IgA production

25   (McGhee, J.R. and H. Kiyono, 1992; Holmgren, J., et al., 1992). In particular, IL-6 has been shown previously to have an adjuvant effect on IgA responses in tears (Pockley, A.G. and P.C. Montgomery, 1991) and Ramsay, et

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_al_. found that humoral immune responses in the
respiratory tract to vaccinia virus-expressed influenza
hemagglutinin were reduced in IL-6 knock-out mice
(Ramsay, A.J., _et al_., 1994). In addition, IL-6

5   over-expression results in enhanced serum IgA responses
(Brandt, S.J., _et al_., 1990).

Along with the dramatic enhancement of protection
from challenge that we observed in the mice that received
HA + IL-6 DNA, we also observed differences in their

10   immune responses compared to the mice that received HA
DNA alone. Virus-specific serum IgA appeared sooner and
IgG and IgA titers were 2-fold higher in the mice that
received HA + IL-6 DNA.

In addition to assessing total virus-specific serum

15   Ab responses by ELISA, we also measured the level of VN
Abs in the serum. The mice that received HA + IL-6 DNA
had a higher VN titer (120 vs. 80) at the time of
challenge.

One intriguing immunologic finding in our study was

20   the presence of virus-specific IgG Ab in nasal washes.
This was the immunologic parameter that differed most
substantially at the time of challenge between the mice
that received HA + IL-6 DNA and those that received HA
DNA alone. Virus-specific nasal IgG was present at a

25   titer of 40 immediately prior to challenge in the HA +
IL-6 DNA mice, whereas nasal IgG was not present in the
mice vaccinated with HA DNA alone until after challenge,
and then only to a titer of 20. The procedure for

obtaining nasal wash samples is very non-traumatic and we
did not encounter bleeding during the collections.
Therefore, we are confident that this IgG does not simply
represent blood contamination at the time of sampling.

5   It is also interesting that virus-specific nasal and
fecal IgA remained below the level of detection in all of
the mice, even after challenge. Immunoglobulin A is the
predominant isotype in mucosal secretions because it is
selectively transported across mucosal surfaces.

10   Immunoglobulin G cannot utilize this same polymeric
immunoglobulin receptor transport system and, therefore,
most likely reaches the mucosal secretions either via
transudation from systemic sources or by an alternative
mechanism following local production. Results from

15   several studies indicate that protection from initial
infection in the upper airway of mice (Bender, B.S., et
al., 1996; Liew, F.Y., et al., 1984; Renegar, K.B and
P.A. Small, Jr., 1991; Renegar, K.B and P.A. Small, Jr.,
1991; Tamura, S., et al., 1991; Takase, H., et al., 1996)

20   and humans (Murphy, B.R. and R.G. Webster, 1996;
Clements, M.L., et al., 1983) correlates with local IgA
responses. However, there is precedent for protection
from influenza virus lethal challenge in mice in the
absence of a mucosal IgA response (Deck, R.R., et al.,

25   1997). To more completely understand the immunologic
basis for the IL-6 adjuvant effect, future studies will
determine whether mice that receive HA + IL-6 DNA are
protected from initial infection in the nasal passages,

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as well as in the lungs, and the role of cell-mediated immune responses.

In summary, gene gun administration of DNA encoding IL-6 induced a very significant adjuvant effect on the
5   protection from challenge infection elicited by an equine influenza virus HA DNA vaccine in mice. We are currently initiating similar studies in horses themselves, as well as in pigs, where influenza virus is also an important pathogen. Immune responses against influenza virus have
10 been demonstrated previously using gene gun-mediated DNA vaccination in both of these species (Swain, _et_ _al_., unpublished results; Lunn, _et_ _al_., unpublished results), but as in our mouse model system, HA DNA vaccination alone has not been sufficient to induce complete
15 protection from challenge. Therefore, we have cloned the porcine IL-6 gene into our vaccine expression vector and demonstrated that it is functionally expressed _in vitro_ (Larsen, _et_ _al_., unpublished results) and have similarly prepared an equine IL-6 clone (see below) to allow us to
20 assess the adjuvant effect of IL-6 on DNA vaccination in these species.

2.   **Co-administration of Plasmid DNA Encoding Human IL-6 with a Swine Influenza Virus Hemagglutinin DNA Vaccine in Mice**.

25 A.   **In General**

We initially demonstrated (as described above) that co-administration of DNA encoding human interleukin-6 (huIL-6) enhances immune responses to an equine influenza virus hemagglutinin DNA vaccine and subsequent protection

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from homologous virus challenge infection in mice with

A/Equine/Kentucky/1/81, an H3N8-subtype influenza virus.

In this section we demonstrate that co-administration of

huIL-6 DNA also enhances protective immunity generated by

5   DNA vaccination against a different influenza virus, an

H1N1-subtype swine influenza virus.

Infection of pigs with H1N1 influenza A virus is an

important problem for the swine industry throughout the

world. Influenza virus infection in pigs can occur as an

10   enzootic problem in a herd, or as explosive outbreaks of

acute respiratory disease with fever, anorexia, lethargy,

weight loss, nasal and ocular discharge, coughing and

fulminant dyspnea. Although rarely fatal, swine

influenza can be of substantial economic impact because

15   of costs for veterinary care and a delay in reaching

market weight (Easterday, B.C. and V.S. Hinshaw, 1992).

Beyond the impact of influenza for the swine

industry, pigs are also very important in the ecology and

evolution of influenza A viruses in humans. The major

20   pandemics of human influenza this century were caused by

strains that were reassortants between pre-existing human

and avian viruses (Webster, R.G., et al., 1992). Because

the respiratory tract of pigs contains receptors for both

avian and mammalian influenza viruses (Ito, T. and Y.

25   Kawaoka, 1998), pigs are uniquely susceptible to

infection with viruses of avian (Guan, Y., et al., 1996;

Kida, H., et al., 1994; Pensaert, M., et al., 1981;

Scholtissek, C., et al., 1983), as well as swine and

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human origin (Nakajima, K., et al., 1982; Ottis, K., et
al., 1982; Shu, L.L., et al., 1994; Zhou, N., et al.,
1996). As such, they have been implicated as
intermediate species for adaptation of avian viruses to

5   mammals (Campitelli, L., et al., 1997) and as the "mixing
vessels" in which human-avian virus reassortment occurs
(Scholtissek, C., et al., 1983; Scholtissek, C. and E.
Naylor, 1988; Webster, R.G., et al., 1992). Most
recently, human-avian virus reassortants have been

10   isolated from commercially-raised pigs in Europe
(Castrucci, M.R., et al., 1993), and subsequently, from
children in The Netherlands (Claas, E.C.J., et al.,
1994). The later point emphasizes the fact that pigs
also pose a threat as direct sources for zoonotic

15   transmission of swine influenza viruses (Dasco, C.C., et
al., 1984; de Jong, J.C., et al., 1988; Hinshaw, V.S., et
al., 1978; Wentworth, D.E., et al., 1997), sometimes
resulting in the death of the people infected (Eason,
R.J. and M.D. Sage, 1980; Kimura, K., et al., 1998;

20   Patriarca, P.A., et al., 1984; Rota, P.A., et al., 1989;
Top, F.H. and P.K. Russell, 1977; Wentworth, D., et al.,
1994).

One potential mechanism for control of influenza
virus infection in pigs is vaccination. Only one

25   inactivated whole virus vaccine is commercially available
in the U.S. Although this vaccine can reduce signs of
illness and virus shedding, it does not provide the
complete protection from infection and clinical disease

that occurs following recovery from natural infection (Brown, G.B. and J.K. McMillen, 1994; Macklin, M.D., et al., 1998). We have, therefore, investigated DNA vaccination as an alternative approach.

5   B.   Materials and Methods

Influenza virus and DNA vaccine preparation. A/Swine/Indiana/18726/88 (H1N1) (Sw/IN), a prototypical swine influenza virus, was obtained from the influenza virus repository at the University of Wisconsin-Madison.

10   This virus was propagated in embryonated chicken eggs as described previously in this application for our equine influenza virus experiments. The HA gene of Sw/IN was amplified by reverse transcriptase-polymerase chain reaction and cloned into pUC18 plasmid (Noble, S., et

15   al., 1993). Thereafter, the HA gene was subcloned into a eukaryotic expression plasmid containing the promoter and intron A of human CMV and the ampicillin resistance gene, creating a swine influenza virus HA DNA vaccine plasmid hereafter referred to as pWRG1683 (Macklin, M.D., et al.,

20   1998). This plasmid is very similar to the equine HA DNA vaccine plasmid described previously in this application, except that it contains the H1 swine influenza virus HA gene instead of the H3 equine influenza virus HA gene, and it contains ampicillin antibiotic resistance instead

25   of kanamycin. The plasmid expressing huIL-6 used in these experiments is that same as that used in our equine influenza virus experiments. All DNA and gold bead

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preparation for gene gun administration was conducted as described previously in this application.

   Vaccination protocol. Three groups of BALB/c mice were established. DNA vaccines were administered by
5 Accell gene gun as described previously in this application. Each mouse was vaccinated twice, on days 0 and 21. One group of mice (n=16) received only 5.0 μg pWRG plasmid DNA and served as controls. The second group of mice (n=16) received 2.5 μg pWRG1683 + 2.5 μg
10 pWRG (HA DNA vaccinates), and the third group of mice (n=16) received 2.5 μg pWRG1683 + 2.5 μg pWRGhuIL6 (HA + IL-6 vaccinates). Two weeks following the second vaccination, 8 mice in each group were euthanized for collection of serum, cervical and mediastinal lymph nodes
15 (CLN and MLN) and spleen samples for immunological assays. The remaining mice were challenge-infected by intranasal instillation of $10^{3.4}$ EID_{50} of Sw/IN. (Note: because Sw/IN is 140-fold more highly infectious for mice than Eq/KY virus (Larsen, D.L., et al., 1998), this
20 amount of virus is approximately equivalent in mouse infectious dose$_{100}$ units [MID_{100}] to the $10^{7.4}$ EID_{50} challenge dose of Eq/KY used in our earlier experiments (27.7 MID_{100} of Eq/KY versus 50 MID_{100} of Sw/IN). Three days following challenge, the mice were euthanized, the amount of
25 infectious virus in their lungs was determined, and serum and tissue samples as listed above were collected for immunological assays.

Virological and immunological assays. Virus
titrations by growth in eggs and detection of virus-
specific antibodies (Abs) in serum by ELISA were
conducted as described previously in this application,

5   with the exception that Sw/IN was used as the target Ag
in ELISA. As an additional measure of immune responses
in this experiment, antibody-secreting cells (ASC) were
enumerated using an enzyme-linked immunosorbent spot
(ELISPOT) assay. This assay allows one to detect the

10   presence of virus-specific B lymphocytes secreting Ab of
the IgG or IgA isotypes in specific locations in the
body. We enumerated ASC in CLN, MLN and spleen. Single
cell suspensions from these organs are prepared by tissue
disruption through a stainless steel mesh screen, after

15   which the red blood cells are lysed with ammonium
chloride and viable cells counted using trypan blue dye.
The cell preparations are then assayed in 96 well plates
on nitrocellulose membranes coated with either 100 HA
units of Sw/IN as specific Ag or 100 HA units of an

20   antigenically unrelated influenza B virus as a negative
control. After blocking the membranes with 5% fetal
bovine serum (FBS), cells are added at concentrations of
10^3 to 10^6 cells/well and incubated on the Ag-coated
membranes overnight at 37°C. In this assay, Abs that

25   virus-specific lymphocytes secrete during incubation bind
to the underlying Ag in a discrete "spot." Following
washing to remove the cells, these spots are detected by
incubation with alkaline phosphatase-conjugated anti-

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mouse IgG or IgA and NTB/BCIP color reagent. The numbers of virus-specific ASC are reported as number of spots/10^6 starting cells.

C.　Results and Discussion

5　　　Consistent with the results of our previous equine influenza virus study, co-administration of huIL-6 DNA and swine influenza virus HA DNA enhanced protection from challenge infection and virus-specific immune responses in mice compared to administration of HA DNA alone.

10　　　*Protection from challenge infection.* HA DNA vaccination alone lead to only a slight reduction in virus titers in the lungs compared to controls, whereas HA + IL-6 DNA vaccination induced a highly statistically significant level of protection (p=0.004, ANOVA

15　　analysis), including 4/8 mice that were completely protected and had no detectable virus in their lungs after challenge. The mean virus titers in the lungs of mice in this experiment (± standard errors of the means [SEM]) are shown in the table below.

20　　　　　　　　　　　　　　　　TABLE 1

Vaccination Group	Mean virus titer (log EID_{50}/g lung tissue) ± SEM 3 days after challenge infection with $10^{3.4}$ EID_{50} of Sw/IN
Control (pWRG alone)	6.68 (0.16)
HA DNA (pWRG1683 + pWRG)	6.14 (0.43)
HA + IL-6 DNA (pWRG1683 + pWRGhuIL6)	2.46 (1.0)*

25　*statistically significant enhancement of protection compared to HA DNA alone (p=0.004, ANOVA)

We observed the following immunologic responses:

(i) Virus-specific serum IgG titers immediately prior to challenge were 2-fold higher in the HA + IL-6

vaccinated mice than the mice that received HA DNA (pWRG1683) alone.

(ii) The numbers of virus-specific IgG ASC in the spleens of the HA + IL-6 vaccinated mice were 4-fold
5   higher at 2 weeks after the second vaccination compared to the mice vaccinated with HA DNA alone (2.1 vs. 0.6 spots/10^6 cells).

(iii) The HA + IL-6 DNA vaccinated mice had detectable virus-specific IgA ASC in their spleen prior
10   to challenge (1.5 spots/10^6 cells), whereas the HA DNA alone mice did not.

(iv) The HA + IL-6 vaccinated mice were primed for a virus-specific ASC response in the MLN after challenge (4.8 spots/10^6 cells), whereas the mice receiving only HA
15   DNA had no detectable virus-specific ASC in their MLN. This ability of the HA and IL-6 DNA co-administration regime to induce virus-specific ASC in the MLN is particularly significant. We (Larsen, D.L. and C.W. Olsen, 1998) and others (Justewicz, D.M., et al., 1995)
20   have previously shown that the MLN is an important site of ASC responses following experimental influenza virus infection in mice, and it is clear that mice that have recovered from such infections are solidly immune to subsequent virus challenge (Larsen, D.L. and C.W. Olsen,
25   1998). In addition, preliminary results indicate that MLN are also a prominent site of virus-specific primary and memory B cell responses following experimental Sw/IN infection in pigs (Larsen, D.L. and C.W. Olsen, 1998).

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Therefore, we are confident that co-administration of Sw/IN HA and porcine IL-6 DNA will also lead to protection of pigs from challenge infection.

5   3.   <u>Co-administration of HA and huIL-6 DNA Enhances Protection Following High-dose Sw/IN Challenge Infection in Mice</u>.

A.   <u>Introduction</u>

As a follow-up to the results outlined in Experiment 2, we conducted a second study to examine whether co-

10 administration of HA and huIL-6 DNA would enhance protection from infection with a dramatically higher dose of challenge infection.

B.   <u>Materials and Methods</u>

Three groups of BALB/c mice were established (n=10

15 mice/group). The mice were vaccinated on days 0 and 21 with a total of 5.0 μg of DNA, either pWRG alone (controls), pWRG1683 + pWRG (HA vaccinates) or pWRG1683 + pWRGhuIL6, and then challenged 2 weeks after the second vaccination with $10^{6.2}$ EID_{50} Sw/IN. (This represents an

20 approximately 3-log increase in challenge level compared to Experiment I, and, based upon prior infectivity results (Larsen, D.L., <u>et al</u>., 1998) is equal to 31,623 MID_{100}.

C.   <u>Results and Discussion</u>

25 With this extremely high dose of challenge virus, all of the mice, regardless of vaccination, became infected and replicated virus in their lungs. Remarkably however, co-administration of HA + IL-6 DNA still

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enhanced the level of protection compared to HA DNA

vaccination alone.   The lungs of the HA + IL-6 DNA

vaccinated mice contained a significantly (p=0.04, ANOVA)

lower amount of virus after challenge compared to the HA

5   DNA alone vaccinates.   The mean virus titers in the lungs

± standard errors of the means [SEM] are shown in the

table below.

TABLE 2

Vaccination Group	Mean virus titer (log EID_{50}/g lung tissue) ± SEM 3 days after challenge infection with $10^{7.4}$ EID_{50} of Sw/IN
Control (pWRG alone)	6.66 (0.32)
HA DNA (pWRG1683 + pWRG)	4.86 (0.42)
HA + IL-6 DNA (pWRG1683 + pWRGhuIL6)	3.33 (0.72)*

*statistically significant enhancement of protection compared to HA DNA alone (p=0.04, ANOVA)

These data indicate that co-administration of IL-6

15 and HA DNA represents a powerful approach to vaccination

that offers benefits over HA DNA vaccination alone even

in the face of an extreme challenge dose of virus.

4.   <u>Use of Equine Il-6 DNA as an Adjuvant to DNA
     Vaccination</u>.

20 A.   <u>Introduction</u>

Prevention of many infectious diseases is critically

dependent on vaccination.   Successful vaccines frequently

mimic natural infection, especially when the disease

naturally induces long lasting protective immunity.   A

25 good example is equine influenza virus, in which

infection induces protective immunity for over 12 months

and is associated with nasal IgA, and serum IgGa and IgGb

antibody isotype responses.   In sharp contrast current

vaccines provide incomplete, short lived protection.

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Therefore, we investigated DNA vaccination of horses with the equine influenza virus hemagglutinin gene. Our results showed complete clinical protection and partial virological protection that were associated with IgGa and

5. IgGb antibody responses in the absence of a mucosal IgA response. Despite the success of this initial DNA vaccination approach, improvement of the strategy remains important. Specifically the complete prevention of viral shedding in clinically protected horses could

10 significantly decrease the viral transmission rate in the horse population. A second generation of DNA vaccines that uses the co-administration of cytokine genes as adjuvants has the potential to enhance immune responses to DNA vaccination alone. Our efforts are focused on

15 interleukin 6, a cytokine with a wide range of activities in acute phase reactions and the regulation of B and T cell functions. In particular IL-6 regulates isotype switching and promotes T-Helper-2 responses, which have a pivotal role in mucosal immunity.

20   We, therefore, proposed the hypothesis that co-administration of an equine IL-6 plasmid will increase the protective immunity resulting from DNA vaccination of horses.

B.   Subcloning and expression of equine IL-6 for
25   gene gun vaccination

Equine interleukin 6 has previously been cloned and sequenced, and was kindly provided to us by Dr. David Horohov, Louisiana State University, Baton Rouge. In

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order to express IL-6 in a mammalian system, it was necessary to subclone the insert out of its original vector (pCREQIL6) into the gene gun expression vector (pWRG1647) that was provided by PowderJect Vaccines. The

5   IL-6 insert was cut out of its original vector at the HindIII and NotI site using HindIII and NotI restriction enzymes and ligated into the pWRG1647 vector at the same sites using T4 DNA ligase.

The newly synthesized ligation product was then

10   transformed into *E. coli* DHL5 and grown overnight in the presence of ampicillin. Colonies were picked the following day and the plasmid DNA was purified using anion exchange resin chromatography (Qiagen). Clones containing inserts of the correct size were identified by

15   a test digest and visualized on an agarose gel. Taq dye terminator cycle sequencing was performed to verify the correct orientation of the insert and exclude clones containing mutations.

In order to test eqIL-6 protein expression in

20   mammalian cells, the plasmid DNA (pWRGeqIL6) was grown up in larger amounts in *E. coli* and purified by anion exchange resin chromatography. Subsequently the plasmid DNA was coated onto gold beads and transfected into CHO cells using the PowderJect® gene delivery device, a

25   biolistic microparticle delivery device. CHO cell supernatants were collected 24 and 48 hours post transfection and stored at -20°C until further examination. As positive control we collected CHO cell

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supernatants from cells transfected with human IL-6 and as a negative control we collected supernatants from CHO cells expressing empty plasmid (pWRG 1647).

The IL-6 activity of the CHO cell supernatants was

5 tested using the B9 murine cell line, the viability of which depends on the presence of IL-6. Assays are performed by incubating B9 cells in the presence of test supernatants for 72 hours, and then measuring cell viability.

10 To confirm the specificity of equine IL-6 for the B9 cell IL-6 receptor, we also performed experiments in which we blocked binding by incubation with an anti-IL-6 receptor monoclonal antibody, prior to the addition of test supernatants. In all experiments we included a

15 positive standard by adding known concentrations of recombinant human IL-6 to the control wells and a negative standard which were B9 cells in media alone. The viability of the B-9 cells was measured by addition of the tetrazolium salt, XTT. Live cells convert XTT to

20 a colored form, which is detectable with a spectrophotometer at 450 nm.

Empty vector supernatants, independent of treatment, did not support B9 cell viability. High B9 cell viability confirms the successful expression of human

25 IL-6 and activity was blocked by prior incubation of B9 cells with anti-IL-6 receptor antibody. The same pattern was seen with supernatants from equine IL-6 transfected CHO cells, and similarly B9 cell viability was reduced by

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the antibody to the IL-6 receptor. These experiments
confirmed the functional activity of the pWRGeqIL-6
vaccine plasmid in mammalian cells.

C.   Adjuvant Activity of Equine IL-6 DNA in DNA
5   Vaccination of Horses with the Equine Influenza
          Virus Hemagglutinin (HA) Gene.

An experiment was carried out using two groups of
seven influenza virus naive ponies, which were maintained
in isolation suites for the duration of the experiment.

10   Both groups of ponies were subjected to DNA vaccination
using the same plasmid expressing the equine influenza
virus HA gene that was used in the mouse experiments
described previously in this application. Horses will be
vaccinated at skin and mucosal (tongue and conjunctal

15   surface of the eye) sites using protocols previously
demonstrated by us to be effective in generating
protective anti-influenza virus IgGa and IgGb antibody
responses.

In one of these vaccination groups the HA DNA was
20   combined with equine IL-6 DNA. In the other group the HA
DNA was combined with a similar amount of DNA consisting
of empty vector as a control. Three vaccinations were
administer at approximately 60 day intervals, and 14 days
after the third vaccination three ponies from each

25   vaccination group were euthanized for measurement of
influenza virus specific lymphoproliferative responses in
regional lymphoid tissues. At the same time
lymphoproliferative responses were measured in the same
tissues in four influenza virus naive ponies, and four

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further ponies that had recovered from an influenza virus
infection fourteen days previously.

A total of 45 days after the third vaccination the
remaining 4 ponies in each DNA vaccination group were

5   subjected to an intranasal influenza virus challenge; an
identical challenge was administered to three
seronegative control ponies.

All seven naive control ponies that were subjected
to an influenza virus challenge showed clinical signs of

10   infection, including fever, coughing, and mucopurulent
nasal discharge. In addition all seven ponies shed virus
for up to seven days post-challenge. None of the four
ponies in either DNA vaccination group showed any
clinical signs of disease after challenge infection. In

15   each DNA vaccination group, only one pony shed virus in
excess of two days post-viral challenge, and in each
instance at lower titers than in the control ponies.
Both DNA vaccination pony groups generated similar strong
influenza virus specific serum antibody responses of the

20   IgGa and IgGb antibody isotypes subsequent to the second
and third DNA vaccinations; no other antibody isotype
responses were detected prior to challenge infection.

Subsequent to challenge infection the two DNA
vaccination groups could be clearly distinguished in that

25   the equine IL-6 DNA adjuvanted group developed a strong
IgG(T) isotypic response far in excess of that generated
by HA DNA vaccination alone. In addition the equine IL-6
DNA adjuvanted group developed a stronger respiratory

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tract mucosal IgA response than that generated by HA DNA
vaccination along. This demonstrates that use of equine
IL-6 DNA as a an adjuvant results in stronger priming of
the equine immune response as evidenced by induction of
5   an additional serum IgG sub-isotypic response, and
increased mucosal IgA responses in response to challenge
infection.

In lymphoproliferative responses a similar
augmentation of influenza virus specific immune responses
10   by use of equine IL-6 DNA. This was seen in the regional
lymph nodes in the inguinal area which drain skin regions
used for administration of DNA vaccination. After
preparation of lymphocytes from these tissues and
stimulation with influenza virus, both DNA vaccination
15   groups showed greater proliferative responses than either
naive control ponies or ponies previously infected with
influenza virus. However, this responses were more than
two standard deviations higher in the ponies administered
the equine IL-6 DNA as a an adjuvant.
20   Overall, the use of equine IL-6 DNA shows strong
evidence of being able to both amplify and modify
influenza virus-specific immune responses in the horse,
and, consequently, may be of considerable value in
improving protective immunity resulting from DNA
25   vaccination in this species.

5.   Co-deliver of DNA Expressing hepsAg (7128) and DNA Expressing Human IL-6 in Mice.

   A.   In General

   In the following experiment we demonstrate that co-
5   delivery of DNA encoding the human hepatitis surface antigen (SAG) a DNA expressing human IL-6 provoked an enhanced CTL response in the mouse model system. These results demonstrate that the DNA vaccination/IL-6 co-administration method may have usefulness in broader
10   applications, including cancer vaccines.

   B.   Materials and Methods

   Expression vectors: The expression vector WRG7128 encodes human hepatitis surface antigen (sAg). WRG7077 is the empty control vector missing the insert sequences
15   encoding sAg. The expression vector encoding human IL-6 (hIL-6) was obtained as above.

   Immunizations: Balb/C female mice (8-10 weeks) were immunized with the PowderJect® XR gene delivery device at weeks 0 and 4. Each mouse received two targets
20   consisting of a co-delivery of two expression vectors into the upper and underside of the tongue or abdominal skin as follows:

   Group 1:   1.25 μg sAg (7128)+ 1.25 μg hIL-6
   delivered to the tongue.
25
   Group 2:   1.25 μg sAg (7128)+ 1.25 μg (7077)
   delivered to the tongue.

   Group 3:   1.25 μg sAg (7128) + 1.25 μg hIL-6
30   delivered to the skin.

   Group 4:   1.25 μg sAg (7128) + 1.25 μg (7077)
   delivered to the skin.

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Group 5 (Negative Controls): 1.25 μg (7077) +
1.25 μg (hIL-6) delivered to either the skin or
tongue.

CTL Assay: A chromium release assay was used to

5   measure the ability of *in vitro*-stimulated splenocytes to

lyse target cells expressing hepatitis surface antigen.

Mice were sacrificed 2 weeks following the final

immunization. Splenocytes were harvested and red blood

cells lysed with ACK lysis buffer (Sigma). Following

10   three washes with RMPI-10 (RPMI-1640 plus 10% heat-

activated fetal calf serum and 50 μg/ml gentamycin),

splenocytes were resuspended to 6 x 10⁶ cells per ml in SM

medium (RPMI-10 plus 1 mM sodium pyruvate plus 1x

nonessential amino acids). Stimulator cells were

15   mitomycin C-treated splenocytes from naive mice pulsed

with a synthetic peptide representing a known hepsAg CTL

epitope (IPQSLDSWWTS). A total of 2.0 x 10⁶ stimulator

cells were cocultivated with 6 x 10⁶ responder cells from

immunized mice in a 24-well culture plate in SM

20   supplemented with 10 U of rat IL-2/ml (Collaborative) for

6 days at 37°C and for 5 hours with 30,000 radiolabelled

target cells (P815 cells transformed with a vector

expressing hepatitis surface antigen) at various

effector/target ratios. Target cell lysis was measured

25   by liquid scintillation counting of 40 μl of cell

supernatants. Percent-specific lysis of labeled target

cells for a given effector cell sample was calculated as

follows:

$$\% \text{ Specific lysis} = 100\% \times \frac{(\text{Cr51 release in sample} - \text{Spontaneous Cr51 release})}{(\text{Maximum Cr51 release} - \text{Spontaneous Cr51 release})}$$

30

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Spontaneous chromium release represents the amount of radioactivity released from target cells in the absence of effector cells. Maximum chromium release represents the amount of radioactivity released following

5 lysis of target cells with 1% Triton X-100. No specific lysis above background levels was observed for the experimental or control effector cells against P815 cells not expressing hepatitis surface antigen (non-specific lysis).

10 C. Results

Table 3, below, demonstrates an increased CTL response with mice immunized with a co-delivery of IL-6 versus mice immunized with a co-delivery including an empty vector.

15 Percent specific lysis at an effector:target ratio of 50/1 was compared in mice immunized with a codelivery of WRG7128 + IL-6 versus mice immunized with a codelivery of WRG7128 + an empty vector (WRG7077). Mice were immunized in the skin or tongue as indicated. A

20 statistical difference between these groups was determined by paired T-test analysis ($p < 0.05$).

TABLE 3

Effector:Target Ratio=50/1

Target Site	WRG7128 + IL-6 (% specific lysis-control lysis)	WRG7128 + WRG7077 (% specific lysis-control lysis)
Skin	49.8	19.7
Skin	48.5	32.5
Skin	27.2	34.0
Tongue	24.9	-7.7
Tongue	30.5	14.0
Tongue	19.2	1.2

To confirm these results, percent specific lysis was also compared between these two groups at an effector:target ration of 17/1. At this E:T, the difference between mice receiving IL-6 versus the empty vector was also statistically significant ($p < 0.01$).

TABLE 4

Effector:Target Ratio=17/1

Target Site	WRG7128 + IL-6 (% specific lysis-control lysis)	WRG7128 + WRG7077 (% specific lysis-control lysis)
Skin	40.5	9.8
Skin	43.1	23.0
Skin	20.2	18.2
Tongue	17.9	-3.5
Tongue	25.9	11.5
Tongue	15.1	1.0

REFERENCES

Aarden, L.A., De Groot, E.R., Schapp, O.L., Lansdorp, P.M., "Production of hybridoma growth factor by monocytes," Eur. J. Immunol. 17:1411-6, 1987.

Anderson, R., Gao, X.M., Papakonstantinopoulou, A., Roberts, M., Dougan, G., "Immune response in mice following immunization with DNA encoding fragment C of tetanus toxin," Infect. Immun. 64:3168-73, 1996.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., "Current protocols in molecular biology," New York: Greene Publishing Associates and Wiley-Interscience, 1989.

Bender, B.S., Rowe, C.A., Taylor, S.F., Wyatt, L.S., Moss, B., Small, P.A., Jr., "Oral immunization with a replication-deficient recombinant vaccinia virus protects mice against influenza," J. Virol. 70:6418-24, 1996.

Brandt, S.J., Bodine, D.M., Dunbar, C.E., Nienhuis, A.W., "Dysregulated interleukin 6 expression produces a syndrome resembling Castleman's disease in mice," J. Clin. Invest. 86:592-9, 1990.

Brown, G.B. and J.K. McMillen, "MaxiVac™-Flu: evaluation of the safety and efficacy of a swine influenza vaccine," Am. J. Trop. Med. Hyg. 50:3-9, 1994.

Campitelli, L., I. Donatelli, E. Foni, M.R. Castrucci, C. Fabiani, Y. Kawaoka, S. Krauss, and R.G. Webster, "Continued evolution of H1N1 and H3N2 influenza viruses in pigs in Italy," Virology 232:310-318, 1997.

Castrucci, M.R., I. Donatelli, L. Sidoli, G. Barigazzi, Y. Kawaoka, and R.G. Webster, "Genetic reassortment between avian and human influenza viruses in Italian pigs," Virology 193:503-506, 1993.

Chow, Y.H., Huang, W.L., Chi, W.K., Chu, Y.D., Tao, M.H., "Improvement of hepatitis B virus DNA vaccines by plasmids coexpressing hepatitis B surface antigen and interleukin-2," J. Virol. 71:169-78, 1997.

Claas, E.C.J., Y. Kawaoka, J.C. de Jong, N. Masurel, R.G. Webster, "Infection of children with avian-human reassortant influenza virus from pigs in Europe," Virology 204:453-457, 1994.

Clements, M.L., O'Donnell, S., Levine, M.M., Chanock, R.M., Murphy, B.R., "Dose response of A/Alaska/6/77 (H3N2) cold-adapted reasortant vaccine virus in adult volunteers: role of local antibody in resistance to

infection with vaccine virus," _Infect. Immun._ 40:1044-51, 1983.

Dasco, C.C., R.B. Couch, H.R. Six, J.F. Young, J.M. Quarles, J.A. Kasel, "Sporadic occurrence of zoonotic swine influenza virus infections," _J. Clin. Microbiol._ 20:833-835, 1984.

Deck, R.R., DeWitt, C.M., Donnelly, J.J., Liu, M.A., Ulmer, J.B., "Characterization of humoral immune responses induced by an influenza hemagglutinin DNA vaccine," _Vaccine_ 15:71-8, 1997.

de Jong, J.C., Paccaud, M.F., de Ronde-Verloop, F.M., Huffels, N.H., Verwei, C., Weijers, T.F., Bangma, P.J., van Kregten, E., Kerckhaert, J.A.M., Wicki, F., Wunderli, W., "Isolation of swine-like influenza A (H1N1) viruses from men in Switzerland and the Netherlands," _Annu. Inst. Pasteur/Virol._ 139:429-437, 1988.

Donnelly, J.J., Ulmer, J.B., Liu, M.A., "Immunization with DNA," _J. Immunol. Methods_ 176:145-52, 1994.

Donnelly, J.J., _et al._, _Life Sci._ 60:163-72, 1996.

Eason, R.J. and M.D. Sage, "Deaths from influenza A, subtype H1N1, during the 1979 Auckland epidemic," _New Zealand Medical Journal_ 91:129-131, 1980.

Easterday, B.C. and V.S. Hinshaw, "Swine influenza," _Diseases of Swine_, In A.D. Leman, B.E. Straw, W.L. Mengeling, S.D. D'Allaire, D.J. Taylor (eds.), Iowa State Press, Ames, p. 349-357, 1992.

Finkelman, F.D., Holmes, J., Katona, I.M., _et al._, "Lymphokine control of _in vivo_ immunoglobulin isotype selection," _Annu. Rev. Immunol._ 8:303-33, 1990.

Fynan, E.F., Webster, R.G., Fuller, D.H., Haynes, J.R., Santoro, J.C., Robinson, H.L., "DNA vaccines: Protective immunizations by parenteral, mucosal, and gene-gun inoculations," _Proc. Natl. Acad. Sci. USA_ 90:11478-82, 1993.

Fynan, E.F., Webster, R.G., Fuller, D.H., Haynes, J.R., Santoro, J.C., Robinson, H.L., "DNA vaccines: A novel approach to immunization," _Int. J. Immunopharmacol._ 17:79-83, 1995.

Geissler, M., Gesien, A., Tokushige, K., Wands, J.R., "Enhancement of cellular and humoral immune responses to hepatitis C virus core protein using DNA-based vaccines augmented with cytokine-expressing plasmids," _J. Immunol._ 158:1231-7, 1997.

Guan, Y., K.F. Shortridge, S. Krauss, P.H. Li, Y. Kawaoka, R.G. Webster, "Emergence of avian H1N1 influenza viruses in pigs in China," <u>J. Virol.</u> 70:8041-8046, 1996.

Hannant, D., Mumford, J.A. and Jesset, D.M., "Duration of circulating antibody and immunity following infection with equine influenza virus," <u>Vet. Rec.</u> 122:125-8, 1988.

Haynes, J.R., McCabe, D.E., Swain, W.F., Widera, G., Fuller, J.T., "Particle-mediated nucleic acid immunization," <u>J. Biotechnol.</u> 44:37-42, 1996.

Hengge, U.R., Chan, E.F., Foster, R.A., Walker, P.S., Vogel, J.C., "Cytokine gene expression in epidermis with biological effects following injection of naked DNA," <u>Nature Genetics</u> 10:161-6, 1996.

Hinshaw, V.S., Bean, Jr., W.J., Webster, R.G., Easterday, B.C., "The prevalence of influenza viruses in swine and the antigenic and genetic relatedness of influenza viruses from man and swine," <u>Virology</u> 84:51-62, 1978.

Holmgren, J., Czerkinsky, C., Lycke, N., Svennerholm, A.-M., "Mucosal immunity: implications for vaccine development," <u>Immunobiol.</u> 184:157-79, 1992.

Ito, T. and Y. Kawaoka. 1998. Unpublished results.

Justewicz, D.M., Doherty, P.C., Webster, R.G., "The B-cell response in lymphoid tissue of mice immunized with various antigenic forms of the influenza virus hemagglutinin," <u>J. Virol.</u> 69:5414-5421, 1995.

Kida, H., Ito, T., Yasuda, J., Shimizu, Y., Itakura, C., Shortridge, K.F., Kawaoka, Y., Webster, R.G., "Potential for transmission of avian influenza viruses to pigs," <u>J. Gen. Virol.</u> 75:2183-2188, 1994.

Kimura, K., Adlakha, A., Simon, P.M., "Fatal case of swine influenza virus in an immunocompetent host," <u>Mayo Clin. Proc.</u> 73:243-245, 1998.

Larsen, D.L., Dybdahl-Sissoko, N.R., McGregor, M.W., Drap, K., Neumann, V., Swain, W.F., Lunn, D.P., Olsen, C.W., "Coadministration of DNA encoding interleukin-6 and hemagglutinin confers protection from influenza virus challenge in mice," <u>J. Virol.</u> 72:1704-1708, 1998.

Larsen, D.L., Dybdahl-Sissoko, N.R., Olsen, C.W., "Comparison of the infectivity of A/Equine/Kentucky/1/81 and A/Swine/Indiana/1726/88 for mice," Unpublished results, 1998.

Larsen, D.L. and C.W. Olsen, "The mediastinal lymph node is a prominent site of virus-specific primary and memory

B cell responses following influenza virus infection in
mice and pigs," Unpublished results, 1998.

Liew, F.Y., Russell, S.M., Appleyard, G., Brand, C.M.,
Beale, J., "Cross-protection in mice infected with
influenza A virus bt the respiratory route is correlated
with local IgA antibody rather than serum antibody or
cytotoxic T cell reactivity," Eur. J. Immunol. 14:350-6,
1984.

Lin, R., Tarr, P.E., Jones, T.C., "Present status of the
use of cytokines as adjuvants with vaccines to protect
against infectious diseases," Clin. Infect. Dis.
21:1439-49, 1995.

Liu, M.A., Ann. NY Acad. Sci. 772:15-20, 1995 (entire
volume was devoted to DNA vaccination).

Lofthouse, S.A., Andrews, A.E., Nash, A.D., Bowles, V.M.,
"Humoral and cellular responses induced by intradermally
administered cytokine and conventional adjuvants,"
Vaccine 13:1131-7, 1995.

Macklin, M.D., McCabe, D., McGregor, M.W., Neumann, V.,
Meyer, T., Callan, R., Hinshaw, V.S., Swain, W.F.,
"Immunization of pigs with a particle-mediated DNA
vaccine to influenza A virus protects against challenge
with homologous virus," J. Virol. 72:1491-1496, 1998.

Madic, J., Martinovic, S., Naglic, T., Hajsig, D.,
Cvetnic, S., "Serological evidence for the presence of
A/equine-1 influenza virus in unvaccinated horses in
Croatia," Vet. Rec. 138:68, 1996.

McGhee, J.R., Kiyono, H., "Mucosal immunity to vaccines:
current concepts for vaccine development and immune
response analysis. In: Ciardi J.E., eds. Genetically
engineered vaccines. New York: Plenum Press, pp. 3-12,
1992.

Mosmann, T.R. and S. Sad, "The expanding universe of
T-cell subsets: Th1, Th2 and more," Immunol. Today
17:138-46, 1996.

Mumford, J., "Progress in the control of equine
influenza." In: Plowright W, Rossdale PD, Wade JF, eds.
Equine Infectious Diseases VI. Proceedings of the Sixth
International Conference. Newmarket: R and W
Publications, 207-17, 1992.

Mumford, J.A., Jessett, D., Dunleavy, U., Wood, J.,
Hannant, D., Sundquist, B., Cook, R.F., "Antigenicity and
immunogenicity of experimental equine influenza ISCOM
vaccines," Vaccine 12:857-63, 1994.

Murphy, B.R. and R.G. Webster, "Orthomyxoviruses," In: Fields BN, Knipe DM, Howley PM, et al., editors. Field's Virology. 3rd ed. Philadelphia: Lippincott-Raven Publishers, pp. 1397-445, 1996.

Nakajima, K., Nakajima, S., Shortridge, K.F., Kendal, A.P., "Further genetic evidence for maintenance of early Hong Kong-like influenza A (H3N2) strains in swine until 1976," Virology 116:562-572, 1982.

Noble, S., McGregor, M.S., Hinshaw, V.S., "Cloning of the full-length hemagglutinin gene of A/Swine/Indiana/1726/88 influenza virus," Unpublished results, 1993.

Noll, A. and I.B. Autenrieth, "Immunity against Yersinia enterocolitica by vaccination with Yersinia HSP60 immunostimulating complexes or Yersinia HSP60 plus interleukin-12," Infect. Immun. 64:2955-61, 1996.

Olsen, C.W., McGregor, M.W., Cooley, A.J., Schantz, B., Hotze, B., Hinshaw, V.S., "Antigenic and genetic analysis of a recently isolated H1N1 swine influenza virus," Am. J. Vet. Res. 54:1630-6, 1993.

Olsen, C.W., McGregor, M.W., Dybdahl-Sissoko, N., et al., "Immunogenicity and efficacy of baculovirus-expressed and DNA-based equine influenza virus hemagglutinin vaccines in mice," Vaccine 15:1149-1156, 1997.

Ottis, K., Sidoli, L., Bachman, P.A., Webster, R.G., Kaplan, M.M., "Human influenza A viruses in pigs: isolation of a H3N2 strain antigenically related to A/England/42/72 and evidence for continuous circulation of human viruses in the pig population," Arch. Virol. 73:103-108, 1982.

Palmer, D.F., Dowdle, W.R., Coleman, M.T., Schild, G.C., "Advanced laboratory techniques for influenza diagnosis," 6th ed. United States Department of Health, Education and Welfare Immunology Series, 1975.

Patriarca, P.A., Kendal, A.P., Zakowski, P.C., Cox, N.J., Trautman, M.S., Cherry, J.D., Auervach, D.M., McCusker, J., Belliveau, R.R., Kappus, K.D., "Lack of significant person-to-person spread of swine influenza-like virus following fatal infection of an immunocompromised child," Am. J. Epidemiol. 119:152-158, 1984.

Pensaert, M., Ottis, K., Vandeputte, J., Kaplan, M.M., Bachmann, P.A., "Evidence for the natural transmission of influenza A virus from wild ducks to swine and its potential importance for man," Bull. World Health Org. 59:75-78, 1981.

Pertmer, T.M., Eisenbraun, M.D., McCabe, D., Prayaga, S.K., Fuller, D.F., Haynes, J.R., "Gene gun-based nucleic acid immunization: Elicitation of humoral and cytotoxic T. lymphocyte responses following epidermal delivery of nanogram quantities of DNA," Vaccine 13:1427-30, 1995.

Pockley, A.G. and P.C. Montgomery, "In vivo adjuvant effect of interleukins 5 and 6 on rat tear IgA antibody responses," Immunology 73:19-23, 1991.

Ramsay, A.J. and M. Kohonsen-Corish, "Interleukin-5 expressed by a recombinant virus vector enhances specific mucosal IgA responses in vivo," Eur. J. Immunol. 23:3141-5, 1993.

Ramsay, A.J., Husband, A.J., Ramshaw, I.A., Bao, S., Matthaei, K.I., Koehler, G., Kopf, M., "The role of interleukin-6 in mucosal IgA antibody responses in vivo," Science 264:561-3, 1994.

Reed, L.J. and H. Muench, "Simple method of estimating 50 percent endpoints," Am. J. Hyg. 27:493-7, 1938.

Renegar, K.B. and P.A. Small, Jr., "Passive transfer of local immunity to influenza virus infection by IgA antibody," J. Immunol. 146:1972-8, 1991.

Renegar, K.B. and P.A. Small, Jr., "Immunoglobulin A mediation of murine nasal anti-influenza virus immunity," J. Virol. 65:2146-8, 1991.

Robinson, H.L., Lu, S., Feltquate, D.M., et al., "DNA vaccines," AIDS Res. Hum. Retroviruses 12:455-7, 1996.

Rota, P.A., Rocha, E.P., Harmon, M.W., Hinshaw, V.S., Sheerar, M.G., Kawaoka, Y., Smith, T.L., "Laboratory characterization of a swine influenza virus isolated from a fatal case of human influenza," J. Clin. Microbiol. 27:1413-1416, 1989.

Sakaguchi, M., Nakamura, H., Sonoda, K., Hamada, F., Hirai, K., "Protection of chickens from Newcastle disease by vaccination with a linear plasmid DNA expressing the F protein of Newcastle disease virus," Vaccine 14:747-52, 1996.

Scholtissek, C., Burger, H., Bachmann, P.A., Hannoun, C., "Genetic relatedness of hemagglutinins of the H1 subtype of influenza A viruses isolated from swine and birds," Virology 129:521-523, 1983.

Scholtissek, C., Burger, H., Kistner, O., Shortridge, K., "The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses," Virology 147:287-294, 1985.

Scholtissek, C. and E. Naylor, "Fish farming and influenza pandemics," Nature 331:215, 1988.

Shu, L.L., Lin, Y.P., Wright, S.M., Shortridge, K.F., Webster, R.G., "Evidence for interspecies transmission and reassortment of influenza A viruses in pigs in Southern China," Virology 202:825-833, 1994.

Singh, G., "Equine influenza 1987, post-epidemic serological study in North India," J. Eq. Vet. Sci. 12:342-4, 1996.

Sun, W.H., Burkholder, J.K., Sun, J., et al., "In vivo cytokine gene transfer by gene gun reduces tumor growth in mice," Proc. Natl. Acad. Sci. USA 92:2889-93, 1995.

Takase, H., Murakami, Y., Endo, A., Ikeuchi, T., "Antibody responses and protection in mice immunized orally against influenza virus," Vaccine 14:1651-6, 1996.

Tamura, S., Funato, H., Hirabayashi, Y., Suzuki, Y., Nagamine, T., Aizawa, C., Kurata, T., "Cross-protection against influenza A virus infection by passively transferred respiratory tract IgA antibodies to different hemagglutinin molecules," Eur. J. Immunol. 21:1337-44, 1991.

Top, F.H. and P.K. Russell, "Swine influenza at Fort Dix, N.J. IV. Summary and speculation," J. Infect. Dis. 136:S376-S380, 1977.

Traub-Dargatz, J.L., Salman, M.D., Voss, J.L., "Medical problems of adult horses, as ranked by equine practitioners," J. Am. Vet. Med. Assoc. 198:1745-7, 1991.

Ulmer, J.B., Donnelly, J.J., Parker, S.E., et al., "Heterologous protection against influenza by injection of DNA encoding a viral protein," Science 259:1745-9, 1993.

Ulmer, J.B., Deck, R.R., DeWitt, C.M., Friedman, A., Donnelly, J.J., Liu, M.A., "Protective immunity by intramuscular injection of low doses of influenza virus DNA vaccines," Vaccine 12:1541-4, 1994.

Ulmer, J.B., et al., Curr. Opin. Immunol. 8:531-6, 1996.

Van Snick, J., "Interleukin-6: an overview," Annu. Rev. Immunol. 8:253-78, 1990.

Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M., Kawaoka, Y., "Evolution and ecology of influenza A viruses," Microbiol. Rev. 56:152-179, 1992.

Webster, R.G., Fynan, E.F., Santoro, J.C., Robinson, H., "Protection of ferrets against influenza challenge with a

DNA vaccine to the haemagglutinin," <u>Vaccine</u> 12:1495-8, 1994.

Wentworth, D., Xian, X., Cooley, A.J., McGregor, M.W., Hinshaw, V.S., Cox, N., "An influenza A (H1N1) virus closely related to swine influenza responsible for a fatal case of human influenza," <u>J. Virol.</u> 68(4):2051-2058, 1994.

Wentworth, D.E., McGregor, M.W., Macklin, M.D., Neumann, V., Hinshaw, V.S., "Transmission of swine influenza virus to humans after exposure to experimentally infected pigs," <u>J. Infect. Dis.</u> 175:7-15, 1997.

Whalen, R.G., "DNA vaccines for emerging infectious diseases: What if"? <u>Emerg. Infect. Dis.</u> 2:168-75, 1996.

Xiang, Z. and H.C. Ertl, "Manipulation of the immune response to a plasmid-encoded viral antigen by coinoculation with plasmids expressing cytokines," <u>Immunity</u> 2:129-35, 1995.

Zhou, N., He, S., Zhang, T., Zou, W., Shu, L., Sharp, G.B., Webster, R.G., "Influenza infection in humans and pigs in southeastern China," <u>Arch. Virol.</u> 141:649-661, 1996.

CLAIMS

We claim:

    1.   A method for eliciting an enhanced immune response against a selected antigen in a mammalian subject, said method comprising administering to said subject a composition comprising a first nucleic acid sequence encoding interleukin-6 and a second nucleic acid sequence encoding the selected antigen, wherein said first and second nucleic acid sequences are operably linked to control sequences which direct the expression thereof in said subject.

    2.   The method of claim 1, wherein the first and second sequences are present in a single nucleic acid construct.

    3.   The method of claim 1, wherein the enhanced immune response is characterized by the generation of an enhanced antibody response against said antigen.

    4.   The method of claim 1, wherein the enhanced immune response is characterized by the generation of an enhanced T-cell response against said antigen.

    5.   The method of claim 1, wherein the composition is a therapeutic vaccine composition.

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6.   The method of claim 1, wherein the composition is a prophylatic vaccine composition.

7.   The method of claim 3, wherein the antibody response is protective.

8.   The method of claim 1, wherein the selected antigen is a viral antigen.

9.   The method of claim 1, wherein the selected antigen is a bacterial antigen.

10.   The method of claim 1, wherein the selected antigen is a tumor antigen.

11.   The method of claim 1, wherein the composition is administered using particle-mediated delivery techniques.

12.   The method of claim 1, wherein the composition is administered to a target skin site in said subject.

13.   The method of claim 1, wherein the composition is administered to a target mucosal surface in said subject.

14.   The method of claim 13, wherein the composition is administered to the tongue.

15.   The method of claim 1, wherein the viral antigen is derived from a virus selected from the group consisting of influenza viruses, rotaviruses, herepes viruses, and hepatitis viruses.

16.   The method of claim 15, wherein the virus is an influenza virus.

17.   The method of claim 15, wherein the virus is a hepatitis virus.
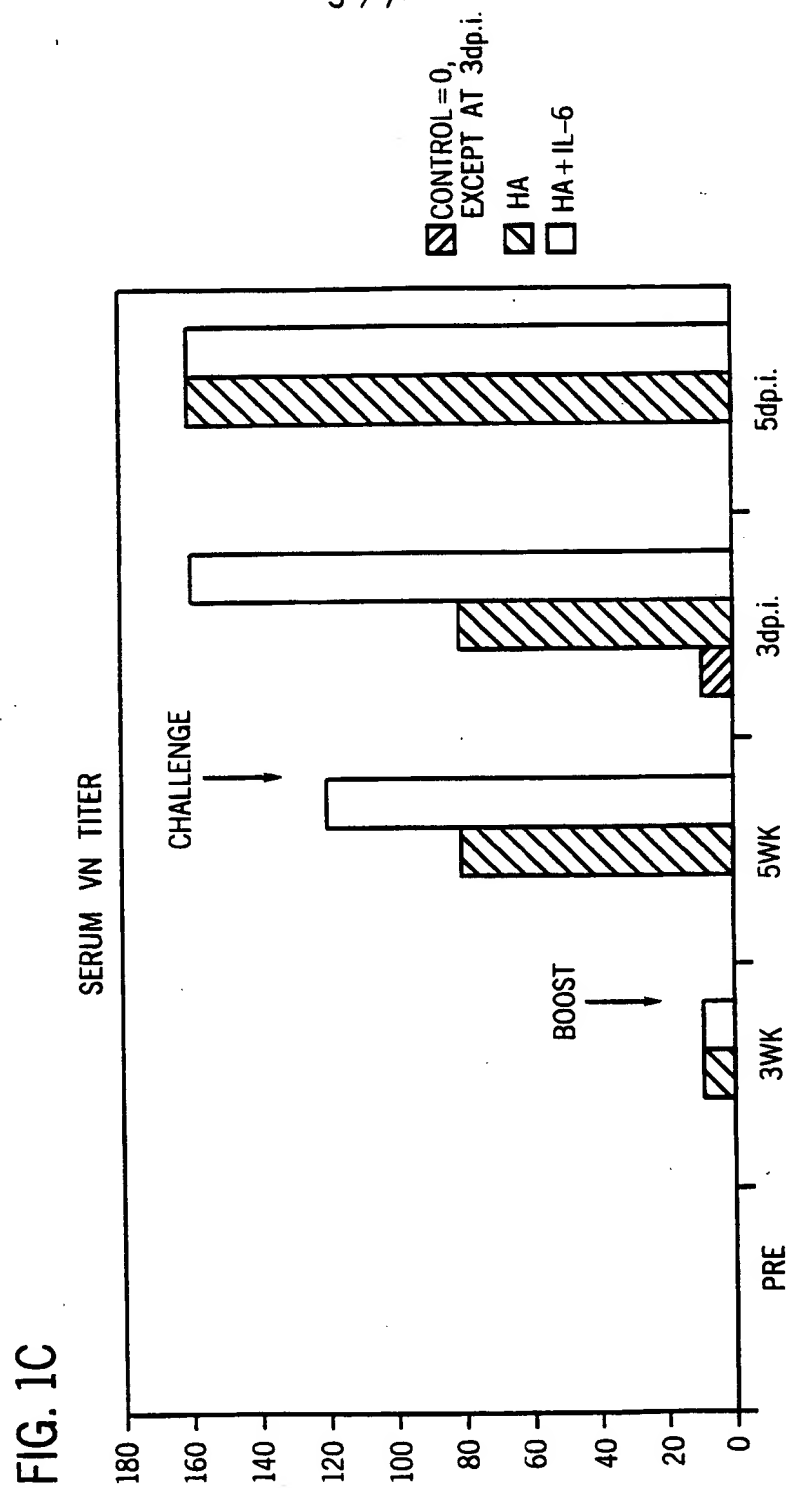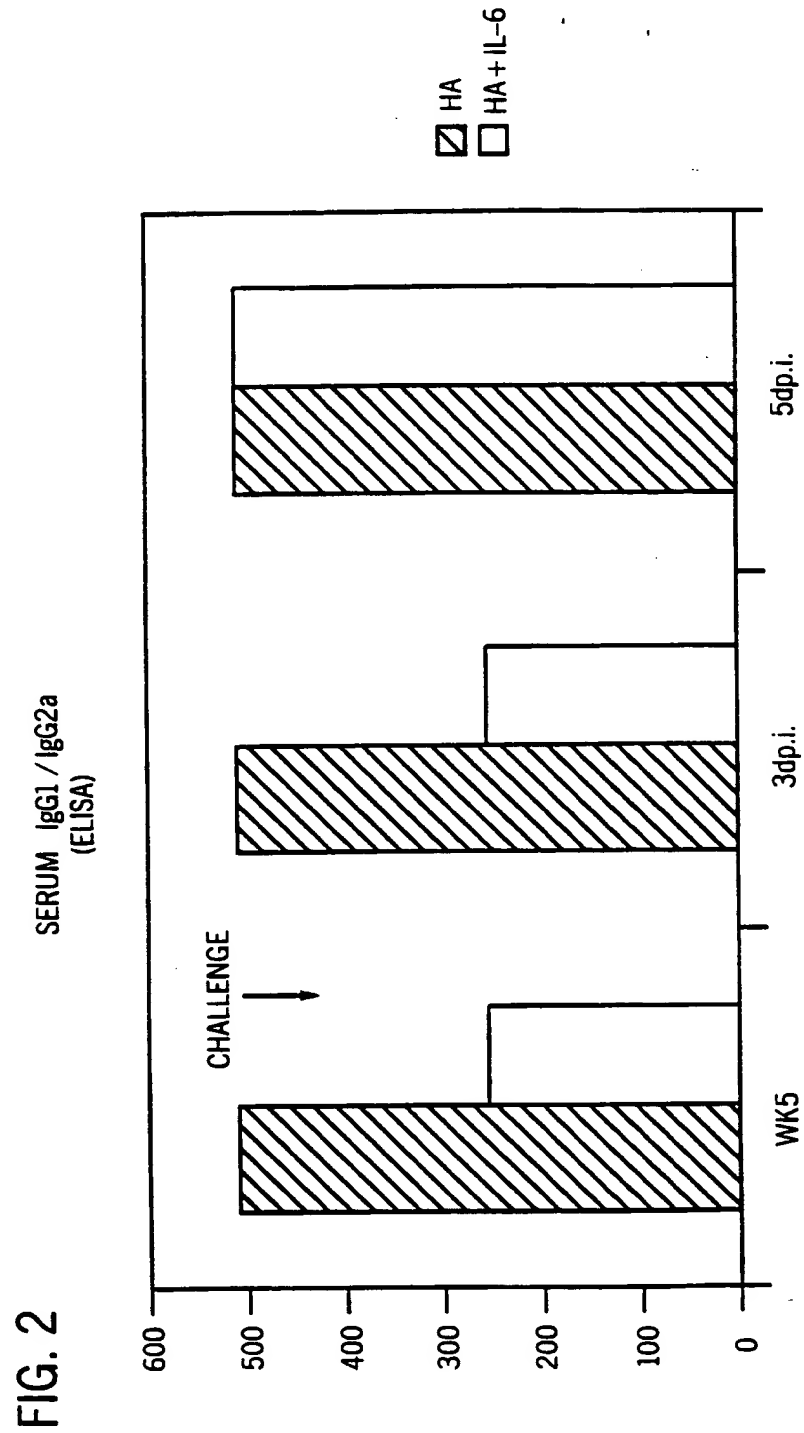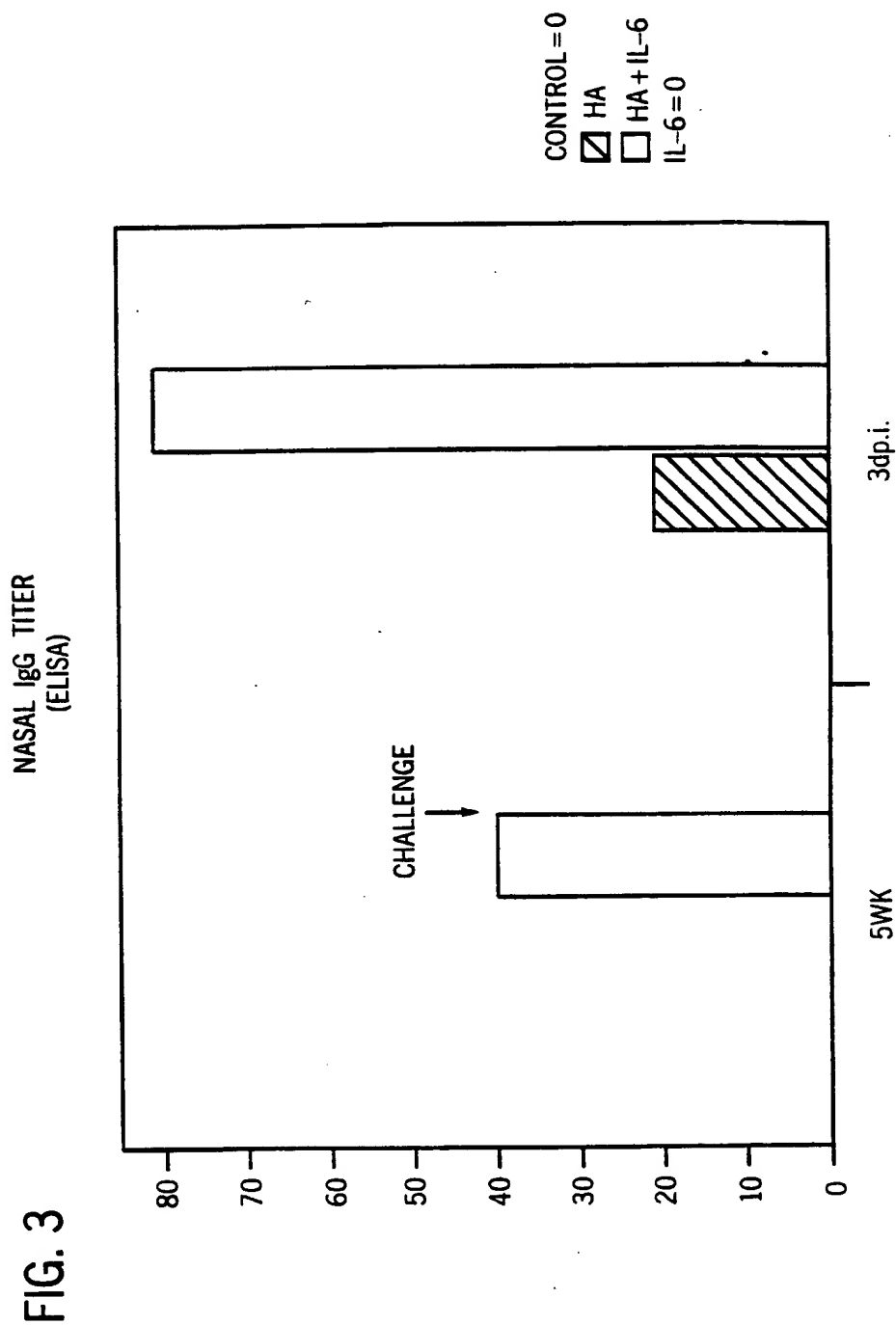
FIG. 1A

SERUM IgG TITER
(ELISA)

CONTROL=0
HA
HA+IL-6
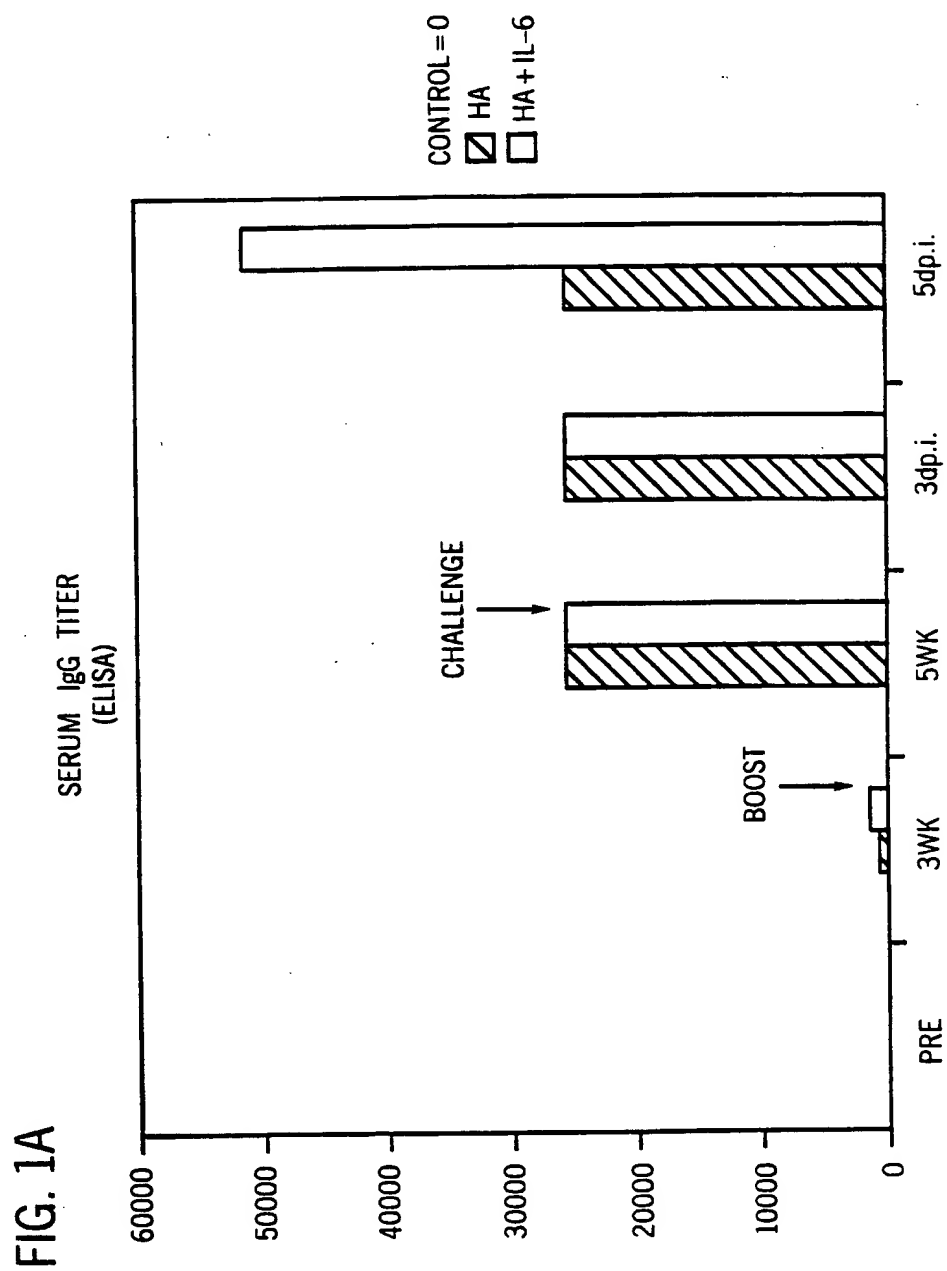
FIG. 1B

SERUM IgA TITER (ELISA)

FIG. 1C

SERUM VN TITER

FIG. 2

SERUM IgG1 / IgG2a
(ELISA)

CHALLENGE

FIG. 3

NASAL IgG TITER
(ELISA)

CONTROL = 0
HA
HA + IL-6
IL-6 = 0

CHALLENGE

3dp.i.

5WK

80
70
60
50
40
30
20
10
0

FIG. 4A

FIG. 4B

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12N15/44   C12N15/51   A61K48/00

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RAMSAY ET AL: "THE ROLE OF INTERLEUKIN-6 IN MUCOSAL IGA ANTIBODY RESPONSES IN VIVO" SCIENCE, vol. 264, 1994, pages 561-563, XP002080400 see the whole document	1-17
X	LINDLEY T ET AL: "Construction and characterization of adenovirus co-expressing hepatitis B virus surface antigen and interleukin - 6." GENE, (1994 JAN 28) 138 (1-2) 165-70. JOURNAL CODE: FOP. ISSN: 0378-1119., XP002080401 Netherlands see the whole document	1-17

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[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 October 1998	27/10/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Sitch, W

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page 1 of 2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	RAMSAY, ALISTAIR J. ET AL: "Enhancement of specific mucosal IgA responses by interleukins 5 and 6 encoded in recombinant vaccine vectors" VACCINES 94: MOD. APPROACHES NEW VACCINES INCL. PREV. AIDS, 'ANNU. MEET.!, 11TH (1994), MEETING DATE 1993, 35-9. EDITOR(S): NORRBY, ERLING. PUBLISHER: COLD SPRING HARBOR LAB. PRESS, COLD SPRING HARBOY, N.Y. CODEN: 60PMAJ, XP002080402 see the whole document ---	1-17
X	RAMSAY A J: "Vector - encoded interleukin-5 and interleukin - 6 enhance specific mucosal immunoglobulin A reactivity in vivo." ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY, (1995) 371A 35-42. JOURNAL CODE: 2LU. ISSN: 0065-2598., XP002080403 United States see the whole document ---	1-17
X	LEONG, KAH HOO ET AL: "Generation of enhanced immune responses by consecutive immunization with DNA and recombinant fowl pox vectors" VACCINES 95: MOL. APPROACHES CONTROL INFECT. DIS., 'ANNU. MEET.!, 12TH (1995), MEETING DATE 1994, 327-31. EDITOR(S): CHANOCK, ROBERT M. PUBLISHER: COLD SPRING HARBOR LABORATORY PRESS, COLD SPRING HARBOR, N. Y. CODEN: 61TGAQ, XP002080404 see the whole document ---	1-17
X	RAMSAY, ALISTAIR J. ET AL: "Enhancement of mucosal IgA responses by interleukins 5 and 6 encoded in recombinant vaccine vectors" REPROD., FERTIL. DEV. (1994), 6(3), 389-92 CODEN: RFDEEH;ISSN: 1031-3613, XP002080405 see the whole document ---	1-17
A	EP 0 538 952 A (YEDA RES & DEV) 28 April 1993 see page 2, line 37 - line 42 ---	10
P,X	LARSEN ET AL: "COADMINISTRATION OF DNA ENCODING INTERLEUKIN-6 AND HEMAGGLUTININ CONFERS PROTECTION FROM INFLUENZA VIRUS CHALLENGE IN MICE" JOURNAL OF VIROLOGY, vol. 72, no. 2, February 1998, pages 1704-1708, XP002080406 cited in the application see the whole document -----	1-17

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INTERNATIONAL SEARCH REPORT

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

   Remark: Although claims 1-17 are directed to a method of treatment of
   the human/animal body, the search has been carried out and
   based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such
   an extent that no meaningful International Search can be carried out. specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application. as follows:

1. [ ] As all required additional search fees were timely paid by the applicant. this International Search Report covers all
   searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee. this Authority did not invite payment
   of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant. this International Search Report
   covers only those claims for which fees were paid. specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently. this International Search Report is
   restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

                           [ ] No protest accompanied the payment of additional search fees.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP 0538952 A	28-04-1993	IL	99821 A	23-07-1996
		CA	2081043 A	23-04-1993